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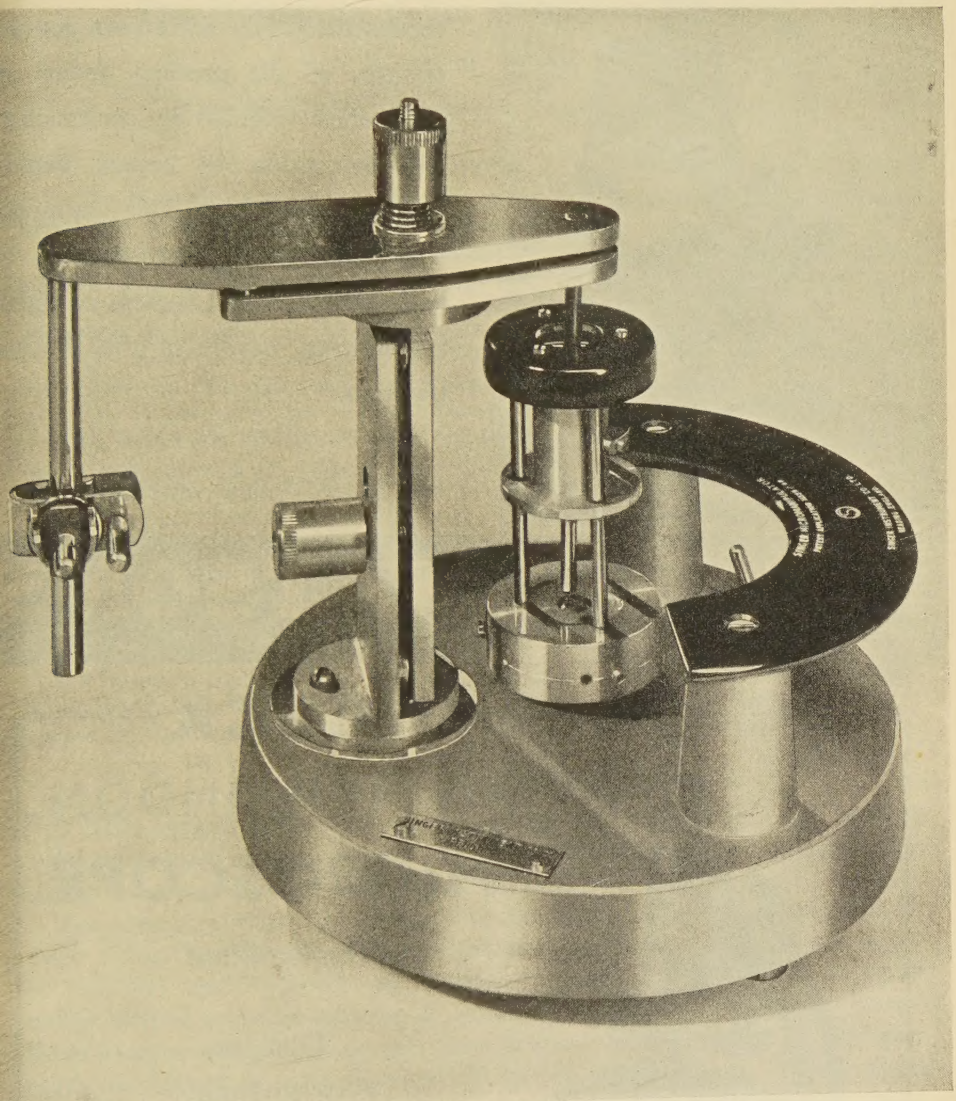

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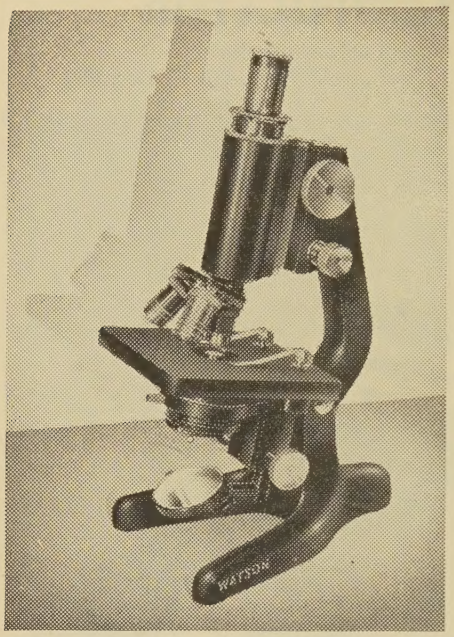

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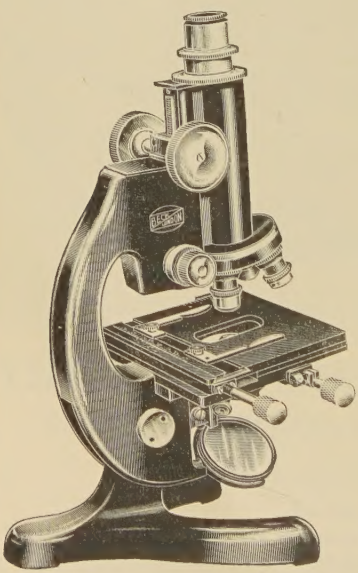

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The Swimming Setae of *Daphnia carinata*

By W. E. AGAR

(*From the Zoology Department, The University of Melbourne*)

Summary

1. The structure, renewal, embryonic development, and regeneration of the swimming setae of *Daphnia carinata* are described.

2. Each seta is formed by four giant cells, two (the core cells) forming the distal segment, and two (the sheath cells) the proximal segment. The inner ends of these cells extend back, as the seta strand, through the whole length of the rami of the antenna to be inserted into the hypodermis half-way down the protopodite.

3. The proximal segment of the seta is formed as an inverted sac, enclosing the distal segment. At ecdysis, the sac is everted.

4. The mechanics of the extrusion of the new setae at ecdysis are described.

5. In embryonic development the seta-forming cells are greatly enlarged hypodermal cells, which grow back from the tip of the antenna to the protopodite.

6. The primary embryonic setae consist of the distal segment only of the adult seta.

7. If a ramus of the antenna is amputated, the missing segments are never regenerated. Formation of new setae takes place, however, from the hypodermal membrane which grows across to close the wound. These setae are formed in the same way as in embryonic development.

8. The muscles in the segment through which the amputation was performed degenerate and are not replaced.

9. The potentiality of enlarging and becoming a seta-forming cell is possessed throughout life by all the hypodermal cells of the two rami of the antenna, but not, apparently, by the cells of protopodite hypodermis.

General Anatomy of the Antenna

THIS work is concerned with the setae of the second antenna of *Daphnia carinata*. There are similar setae on the thoracic appendages, and a pair of them at the posterior bend of the abdomen.

The antenna consists of a protopodite carrying a dorsal and a ventral ramus. Each ramus has three segments, which will be referred to as T (terminal), M (middle), and B (basal). In addition the dorsal ramus has a very small wedge-shaped segment between B and the protopodite. Each ramus carries three *terminal setae*. In the dorsal ramus, segment M, and in the ventral ramus segments M and B, carry a single seta on the ventral border of their distal ends. These will be referred to as the *lateral setae*. Thus there are four swimming setae on the dorsal and five on the ventral ramus. Individual setae will be referred to as S1–5 as in Text-fig. 1A. The length of a seta is about equal to the combined length of the three segments of the ramus.

Each seta is divided by a joint which allows ventral flexion of the distal on the proximal segment. The joint is situated below the middle of the seta, the lengths of the distal and proximal segments being in the ratio of about

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1·25 : 1·00. The greater length of the distal segment is an essential part of the mechanism of extrusion of the new seta at ecdysis.

At its base, the seta is very slightly enlarged to form the *insertion bulb*, which constricts to a very narrow opening at its insertion into the antenna. This bulb, and especially its constricted basal opening, also plays an essential part in the extrusion of the new seta at ecdysis.

Each seta is provided with two lines of fine hairs, one on its dorsal and the other on its ventral surface, the dorso-ventral plane being reckoned with the antenna extended in the antero-posterior axis of the body, and the setae parallel with the antennar axis. A longitudinal line of similar hairs runs almost the whole length of the ventral ramus, but on its dorsal border only. These hairs are absent from the dorsal ramus. In addition, the whole surface of the antenna is closely set with fine pointed spines.

Immediately after ecdysis, the centre of the seta is occupied by a protoplasmic axial fibre, which can be traced almost to the tip of the seta. Later in the instar, this fibre is restricted to the lowest third of the proximal segment.

The complicated muscular system of the protopodite has been described by Klotzsche and by Binder. We, however, are only concerned with the muscles of the two rami. These can be flexed, in the ventral direction only.

As is common in Arthropods, the antennar muscles consist of a strand of sarcoplasm with a bundle of striated contractile fibres running along one side of it.

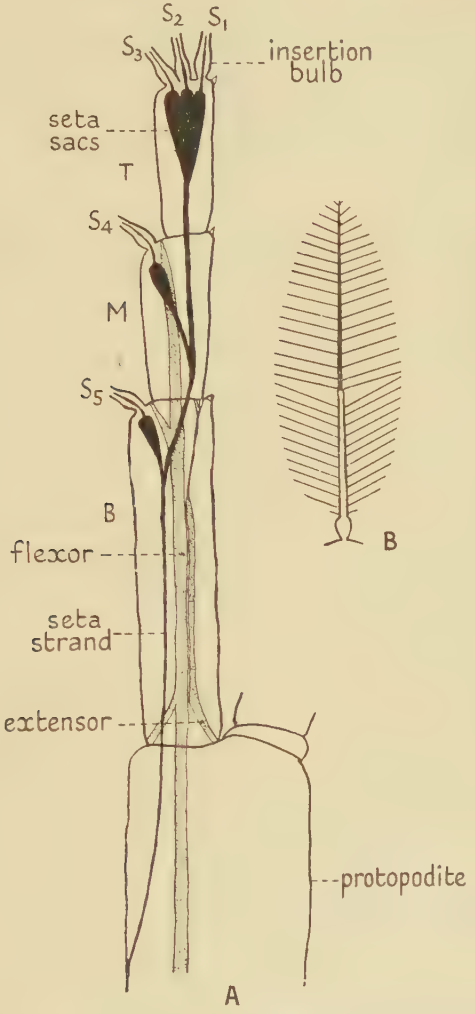
In segment B of the ventral ramus there are two muscles, a larger flexor and a smaller extensor (Text-fig. 1). The sarcoplasmic portions of these are closely applied together. At its distal end the extensor is inserted by a long tendon near the dorsal margin of the joint between B and M. Near the distal end of B, the flexor divides into two, one branch being inserted between the origins of S_5 and segment M; the other branch runs on into M, to be inserted in the corresponding position at the end of that segment. The extensor does not enter M, nor does the flexor enter T. The latter segment is therefore devoid of muscles.

The musculature of the dorsal ramus differs from that of the ventral in that there is no extensor muscle.

The details of the movements of the antenna in swimming are too complicated to make out without photographic aid. We need only concern ourselves, however, with the movements of the setae. These are of two kinds: (1) a flexion of the distal on the proximal segment at the joint between them; this takes place in the ventral direction only; (2) a movement of the seta as a whole at its insertion into the antennar cuticle.

During the swimming stroke the segments of the rami, and of the individual setae, are in extension; the lateral setae stand out at right angles to the antennar axis and the terminal setae are in abduction like a hand with the fingers straightened and spread apart. The whole seta apparatus forms a fan-like pattern presenting the maximum resistance to the water. During the forward stroke, which is in preparation for the next swimming stroke, the segments of the rami are flexed, and the seta fan-work is collapsed; the lateral setae rotate

to a position nearly parallel with the antennar axis (their tips pointing forwards), the terminal setae are no longer spread apart but form a loose bunch, and each individual seta is flexed at the joint between the two segments. These



TEXT-FIG. 1. A. The left ramus of the antenna showing seta strands, muscles, and the bases of the setae. The setae and the segments of the antennae are designated as in the text. B. A seta at half the magnification of A, showing the joint in the axis and the two opposite lines of hairs. These are actually much finer and more numerous than shown in the figure.

movements of the setae, which can be induced by manipulation of an isolated antenna with a needle, are brought about partly by the resistance of the water and partly, in the case of the lateral setae, by their mode of insertion into the antenna. As can be seen in Text-fig. 1A, it follows from the mode of insertion of the flexor muscles that their contraction to flex the segments of a ramus on

each other (as in the forward stroke) will pull down the cuticle between the setae and the points of attachment of the next antennar segments, thus causing the setae to take up a position pointing forwards, and nearly parallel to the antennar axis. There is no direct insertion of muscles into the setae as was erroneously stated in my paper of 1930.

A nerve runs up each ramus of the antenna (Text-fig. 2). Near the tip of segment T it divides into three branches which run into the bases of the terminal setae. A little lower down, about the middle of T, ganglion cells are inserted on its course, usually arranged in an upper group of three, and a lower one of two, cells.

In the dorsal ramus only, a twig from the main nerve, with two ganglion cells on it, runs to the hypodermis at the base of the short spine on the antero-dorsal tip of segment B. This must be interpreted as a sense organ.

I have been unable to find any nerves to S4 or S5 even in preparations where the nerve trunk and its branches to S1–3 and to the sense organ on segment B of the dorsal ramus are all very clear.

Formation and Renewal of the Setae

The seta is produced by four giant cells, each more than a millimetre long in a large *Daphnia*, and with correspondingly large nuclei. Two of these cells are concerned with the formation of the proximal segment of the seta. These we shall call the *sheath cells*. The other two produce the distal segment, and will be referred to as *core cells*. It is probable, however, that these latter, though originating as two hypodermal cells, coalesce during embryonic development into a single binucleate cell.

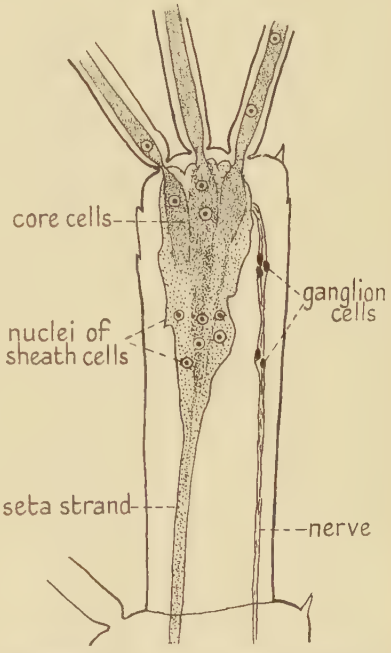
Shortly after ecdysis, the arrangement of these cells is as follows (Text-fig. 4c). The two core cells (or single binucleate cell) form a protoplasmic fibre extending forwards to the tip of the antenna and backwards into a sac, the seta sac, situated just below the base of the seta. The seta sac, which is formed by the two sheath cells, is at this stage a shallow pocket, which will deepen during the course of the instar. At its base, the sheath and core cells taper, and fuse into a thread which we shall call the *seta strand*. This runs back through all the segments of the ramus into the protopodite.

The three seta strands of the terminal setae are often distinguishable from one another to about the base of segment T, but below that they are fused into a single strand showing no evidence of its tripartite formation. The seta strands from S4 and S5 also unite with the strand from the terminal setae (Text-fig. 1) so that a single seta strand from each ramus enters the protopodite. Sections show that the seta strands end by attachment to the hypodermis about half-way down the protopodite.

The terminal segment of the antenna immediately after ecdysis as seen in whole mounts is shown in Text-fig. 2. The nuclei of the core-cells may be situated within the seta itself, or below it in the seta sac. The six nuclei of the three sheaths are below the level of the core nuclei. Transverse sections show the three closely applied sheaths, often very irregular in outline, each with

two nuclei and enclosing a protoplasmic core, which is the core-cells, or binucleate single core cell. The enormous nuclei, both of core and sheath, make them easily distinguishable from any other nuclei in the antenna.

Immediately after ecdysis there begins a withdrawal of the central protoplasmic fibre of the seta, doubtless due to the deepening of the seta sac which progresses throughout the instar. This withdrawal can easily be observed in those cases where one or both of the core nuclei have been drawn into the seta at ecdysis. In 174 setae fixed within 30 minutes after ecdysis, 76 had one or both nuclei within the seta. In 90 setae fixed within the limits 3–5 hours after ecdysis, only one still had a nucleus in it, the other 179 nuclei being now below the tip of the antenna in the seta sac.



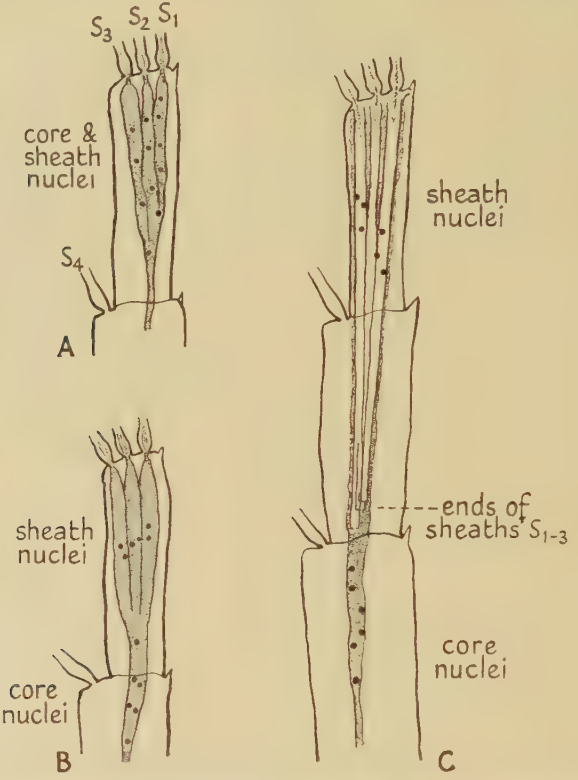
TEXT-FIG. 2. The terminal segment, with bases of the setae, immediately after ecdysis.

Observations of the backward movement of the core nuclei were also made on living animals. One *Daphnia* was anaesthetized in 5 per cent. ether and kept under continuous observation, beginning 20 minutes after ecdysis. In the first 6 minutes two nuclei close together in one seta moved back (towards the base of the seta) through a distance of 16μ. In the next 5 minutes they moved back another 8μ. In another experiment seven *Daphnia*, all large adults, were anaesthetized 10–30 minutes after ecdysis. Those setae which had nuclei in them were selected for observation and the distances of the nuclei from the base of the setae were measured. The animals were then liberated, and the process repeated at intervals of half an hour. In thirty-nine setae so observed, the mean backward movement of the nuclei was 26μ in the first hour and 13μ in the second hour.

About 24 hours after ecdysis the axial protoplasmic fibre only extends about one-third of the way up the proximal segment of the seta, the parts above this being devoid of a protoplasmic axis. The disappearance of the fibre is partly due to its withdrawal as described, and partly, apparently, to its disintegration in the upper part of the seta. The part of the fibre that remains in the lower part of the seta becomes much finer, and surrounded by a chitinous cuticle, with two longitudinal lines of hairs, distinct from the cuticle of the seta itself. This is, in fact, the tip of the new seta, enclosed within the base of the old one. No further withdrawal takes place. The mechanics of the extrusion of the new seta at ecdysis, to be described later, furnish an explanation of

this fact. The long hairs now projecting from the surface of the new seta present sufficient frictional resistance to prevent further withdrawal through the narrow opening at the base of the insertion bulb.

The further deepening of the seta sacs and backward elongation of the core is most easily followed by observation of the movements of the very conspicuous nuclei of the sheath and core cells. As the seta sac deepens and



Text-fig. 3. Three stages in the development of the seta sacs of the terminal setae, shown by the backward migration of the core nuclei relatively to the sheath nuclei. The seta strands from S4 and S5, which join the composite strand from S1–3, are not shown, nor their core and sheath nuclei.

the core therefore lengthens, there is a slight backward movement of the sheath nuclei, but a much greater movement of the core nuclei. These are at first in front of the sheath nuclei (Text-figs. 2, 4C) but travel back along the seta strand so as to lie far behind them (Text-figs. 3C and 4A). In the case of the terminal setae, these nuclei move back into segment B; in a large *Daphnia* this involves a backward movement of a millimetre from their original position. The core nuclei of the lateral setae move back along their seta strands into the protopodite.

During the instar, the sheath cells secrete a layer of chitin on the inner surface of the sheath, forming a cylinder enclosing the seta core. This sheath is

the inverted proximal segment of the new seta. With the deepening of the seta sac this chitinous sheath grows back, finally extending to below the middle of segment M in the case of the terminal setae. This corresponds to the length of the proximal segment of the new seta; its lower end marks the joint between the two seta segments (Text-figs. 3C and 4A). The final condition, just before the next ecdysis, is shown in Text-fig. 4A. The seta core is now completely invested with chitin down to the base of the seta sac, where the cuticles of core and sheath are continuous. Below this, the tapering ends of the sheath and core cells are continued back as the seta strand.

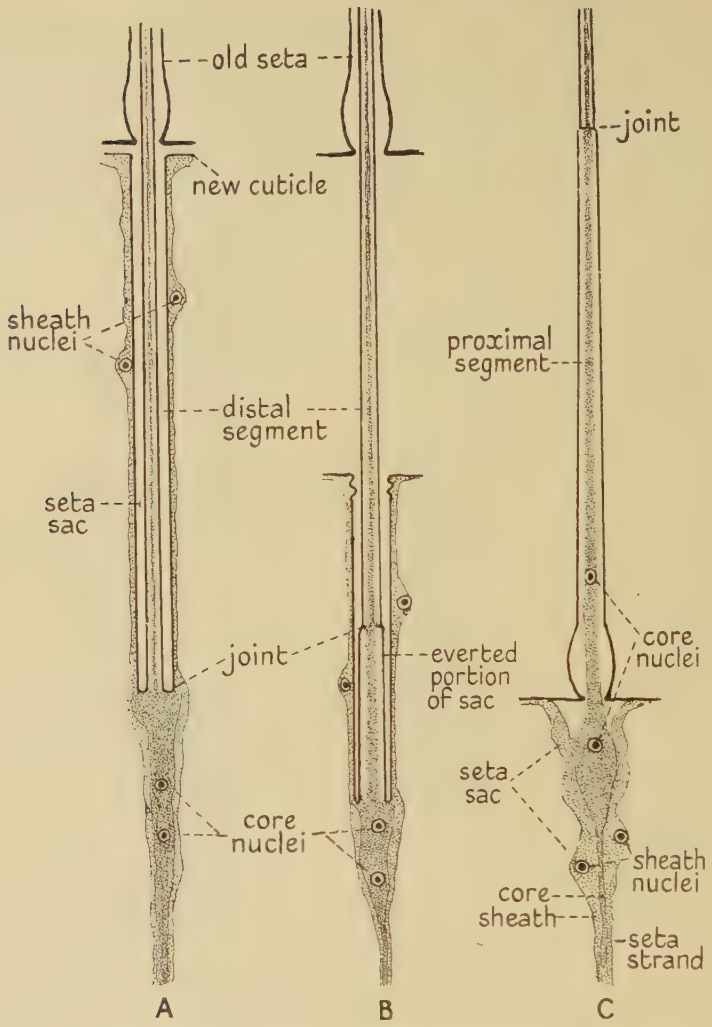
To avoid confusing the figures, the two lines of hairs on the setae are not shown. They are, however, already present in Text-fig. 4A, on the outside of the seta core and on the inside of the sheath. On both core and sheath the hairs pointed forwards. When the seta sac is everted, the hairs turn to approximately a right angle with the seta axis.

The mode of extrusion of a new seta is shown in Text-fig. 4.

Ecdysis starts with the splitting of the head cuticle on each side along a line running from the rostrum dorsalwards between the eye and the antenna to the mid-dorsal line. The head cuticle in front of this line is detached separately from the rest of the cuticle. The carapace then splits along the mid-dorsal line for about the anterior two-thirds of its length. Now the animal pulls itself out of its old cuticle, withdrawing the antenna through the old antennar cuticle, which remains intact. In the extrusion of the new setae, their tips projecting into the old ones play a vital part. As the antenna is withdrawn from its old cuticle, this tip has to be pulled out of the old seta, and therefore through the very narrow constriction at its base. The two lines of fine hairs with which the seta is provided, and which find room to spread slightly in the small terminal expansion of the seta (the insertion bulb), evidently offer sufficient frictional resistance to withdrawal to cause the new seta to be pulled out of its sac as the antenna is withdrawn from its old cuticle. This causes the eversion of the sheath to form the proximal segment of the new seta (Text-fig. 4B). During this progress there is often a corrugation of the upper end of the sheath.

That this is the mechanics of the extrusion of the seta was shown by amputating setae at their bases, so as to remove the insertion bulb, about an hour before ecdysis. When this is done, the new seta is not extruded at all, or sometimes only partly extruded, though unoperated setae on the same antenna are pulled out of their seta sacs in the usual way. This operation was also valuable as a means of getting partially extruded setae (as in Text-fig. 4B), for attempts to get setae in this state by fixing animals in the act of ecdysis were seldom successful, owing to the quickness of the withdrawal of the antenna from its old cuticle.

Setae may be artificially everted from amputated antennae in the following way. The animal is killed shortly before ecdysis is due (between liberation of the young from the brood-pouch and the ecdysis which usually follows within an hour or so). The terminal setae of the amputated antenna are held against the microscope slide, and by means of a needle inserted into the cut base of



TEXT-FIG. 4. Extrusion of a seta at ecdysis, somewhat diagrammatic. The hairs on the seta are not shown. A. Immediately before ecdysis. B. During ecdysis. The fixation of the tip of the new seta in the base of the old one is causing the eversion of the seta sac as the new antenna is withdrawn downwards out of its old cuticle. C. Fully extruded seta. Of the distal segment only the lower portion is shown.

the antenna, it is pulled out of its old cuticle. By this means the new setae are everted from their seta sacs—often completely, sometimes only partially. This operation can be successfully performed on antennae amputated from animals that have been in a 5 per cent. solution of ether for 2 hours after the heart has stopped beating, and in which therefore the tissues must certainly be dead.

At the extrusion of the seta, the protoplasm of the core cells, being continuous with the axial fibre of the distal seta segment, is drawn into the sheath (proximal segment of the seta) as it is everted.

The pulling forward of its anterior end results in a thinning out of the lower part of the seta strand, since its base is fixed in the protopodite. The two core nuclei are also, of course, pulled forward. Their final position depends on how far below the lower end of the seta sac they were situated before ecdysis (Text-fig. 4A). In the fully everted seta they may both be situated in the now shallow seta sac at the tip of the antenna; or if, before the eversion started, they were higher up, one or both may be drawn into the sheath and so come to lie in the proximal segment of the seta itself (Text-figs. 2, 4C). When this occurs they are soon withdrawn below the tip of the antenna as already described.

Earlier Accounts of the Setae

The formation of setae in pockets from which they are extruded in ecdysis has been described for many groups of Crustacea. In Branchiopods, Claus (1876) gives beautiful figures of the setae on the thoracic appendages of *Daphnia similis*. These setae and the pair of backwardly projecting abdominal setae are, as I have verified in *D. carinata* also, of similar structure to those on the antenna. Claus does not, however, describe their formation or renewal at ecdysis.

The most recent account of the setae of a Branchiopod which I have found is that given by Nowikoff for *Limnadia*, a form not, however, very closely related to *Daphnia*. Nevertheless, the structure and formation of the setae are evidently very similar in the two genera. His description of the formation of a new seta, and its extrusion at ecdysis, is in essentials similar to the account I have given for *Daphnia*. He does not, however, describe the mechanics of the extrusion. Moreover, his figure 50 is similar in essentials to my Text-fig. 4A, but he interprets what I have called the seta strand as a nerve, continued into the seta itself. In the position of my two core nuclei are four cells which he interprets as sensory cells. The sheath cells are, to judge from his figures, ordinary hypodermal cells. In fact, the sheath is an invagination of the ordinary hypodermis.

The seta strands and their connexion with the setae have been interpreted by some workers as muscles, or at least as tendons (e.g. Binder, for *D. magna*). In my statistical studies of the regeneration of the antenna (1930, 1931), which were not directly concerned with the histology of the process, I made the same mistake.

Early in an instar, when the seta sacs are depleted and crowded close up under the bases of the setae, the long fine seta strand into which they are continued certainly suggests a nerve or tendon. But later, when the seta sacs have extended down it, it has no such resemblance.

The histology of the seta strand close below the seta sac is shown, somewhat diagrammatically, in Text-figs. 2 and 4. It consists of a central darker staining core, continuous with the core cells, surrounded by a paler sheath continuous with the sheath cells. Lower down, the central core is not always recognizable, and sometimes appears broken up into fibrils. Near its lower end, within the protopodite, the seta strand stains much less strongly and is distinctly fibrillar.

The seta strand is clearly a backward prolongation of the core and sheath cells, and indeed the formation of the strand by backward growth of these cells, derived from the hypodermis, can be followed in embryonic development and regeneration. Its function is to act as a support down which the seta sacs can extend. Fixed as it is at its lower end, when the seta sacs are everted and drawn up to the bases of the setae at ecdysis, it remains as a thin strand, which thickens as the seta sacs spread down it again to form the setae for the next instar.

The mode of formation of bristles in insects (Wigglesworth, 1933, and others) indicates the way in which the complex setae of *Daphnia* and other Crustacea may have evolved. The insect bristle is formed from two cells, a lower hair-forming cell and an upper socket-forming cell. The latter forms a chitinous ring through which a process grows out from the hair-forming cell to form the projecting part of the bristle.

It is easy to see how a sinking down of the hair-forming cell could drag down the socket cell to form a sheath which is everted at ecdysis. In that case, the distal segment of the *Daphnia* seta corresponds to the projecting part of the insect's bristle, and the proximal segment to the elongated and everted socket.

Embryonic Development of the Setae

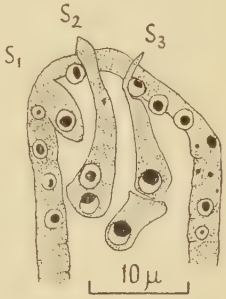
The egg is in a very fluid state when laid. It has the appearance of being poured through the narrow opening of the oviduct into the brood-pouch. Within a few minutes it changes from an irregular sausage shape into a sphere, and at the same time the egg membrane, excessively thin and flexible before this, thickens to form an elastic transparent membrane. About 2 days later (at 'room temperatures') this membrane splits and eclosion of the embryo takes place. The embryo, however, is still enclosed in a very fine transparent membrane, which is not a cuticle in the ordinary sense, for it does not follow the contours of the developing appendages. It must form as a complete internal lining to the egg membrane at a very early stage of development. We shall refer to this membrane as the embryonic membrane.

At the time of eclosion, the rudiments of the antennae have their apices directed backward, slightly pushing out the embryonic membrane from the body. They elongate backwards, between the body and the membrane, till their tips have reached nearly to the hind end of the body. The antennae then rupture the membrane and thus become free. This is brought about by movements of the antennae themselves, which are pulled forward between the body and the membrane, at the same time bending outwards at the joint between the protopodite and the rami. After about half an hour of spasmodic movements of this kind, the membrane is ruptured and the antennae pulled out of it.

After a lapse of a few minutes to an hour or so after their release from the brood-pouch, the embryos undergo an ecdysis which brings the antennar setae into their definitive adult condition.

About 1½ hours after eclosion each seta is represented by an elongating hypodermal cell, its distal end level with the outer boundary of the hypodermis, but internally projecting far below it. These cells are conspicuous, not only for their length, but also for their more densely staining cytoplasm. Owing to the compactness of the tissues at this stage it is difficult to discriminate between the nuclei of these seta cells and those of the hypodermal cells between which they lie.

Text-fig. 5 is of a longitudinal section of the terminal segment of an antenna 4 hours after eclosion, the rudiment of S1 being only just grazed by the razor. Each of the rudiments of S2 and S3 consists of two very large cells, one behind the other, possibly already fused in S2. These are evidently the future cores cells. Their nuclei are already larger than those of the cells of the general hypodermis, and their tips project beyond its surface. This is a projection of the cell itself, not of cuticle. Indeed at this stage no cuticle can be detected. It is not yet possible to identify sheath cells.



Text-fig. 5. Longitudinal section of the tip of an antenna of an embryo 4 hours after eclosion.

By the time the antennae free themselves from the embryonic membrane (about 36–48 hours after eclosion) the rudiments of the three terminal setae form together a multicellular mass, which, as evidenced by the number of nuclei, consists of the full complement of core and sheath cells. Cell boundaries cannot, however, be made out satisfactorily. Posteriorly, this cell mass has already grown back to the protopodite. Anteriorly, the short blunt projections of the core cells in Text-fig. 5 have elongated into fine threads covered with cuticle. These primary setae, as they may be called, correspond to the distal segment of the definitive seta. Thus they lack the joint of the adult seta, but are flexible enough to bend into U- or S-shapes.

The primary setae continue to elongate till by the time the young are released from the brood-pouch (about 5 days after the eggs are laid) they have about half the length of the ramus. Each consists of a staining central strand continuous with the protoplasm of the cell and surrounded by soft cuticle. By now the seta sacs have developed, and the whole structure is similar to that in the adult, except that the projecting part of the seta corresponds to the distal segment only of the adult seta. At the ecdysis which follows release from the brood-pouch, eversion of the sheath takes place as described for the adult, and the animal is now provided with jointed setae of the adult type.

The advantage of this type of swimming seta, consisting of two rigid segments bending at the joint between them in one direction only, over the unjointed but flexible setae of the late brood-pouch young, is evident from the sudden change in the nature of the movements of the animal after this ecdysis. Before this, in spite of the violent action of the antennae the movements of the newly released young are very feeble and result in little true locomotion, in striking contrast to the strong, directed movements immediately after the ecdysis.

Regeneration of the Setae

I have dealt with the regeneration of the antenna in *Daphnia* and *Simocephalus* in two former papers. That of 1930 was a statistical study of the number and length of setae regenerated, and the factors influencing this. The second paper (1931) demonstrated that amputation and subsequent regeneration for a hundred generations had no detectable influence on either the regeneration or normal growth of the antenna. Neither of these papers dealt with the histology of the regeneration process.

As stated in the earlier papers, and confirmed by the present work, the segments of the antenna removed by the operation are never regenerated; new setae, however, are formed freely from the tip of the antennar stump after amputation through any one of the segments T, M, and B.

One conclusion of my former papers needs to be modified by the results of the new work. In the earlier experiments the dorsal ramus of the right antenna was amputated within a few hours of release from the brood-pouch, through segment B in *Daphnia* and M in *Simocephalus* (designated in the earlier papers as II and III respectively). Thus four setae, three terminal and one lateral, were removed in each case. The number and length of setae regenerated were recorded in the first adult instar. In more than a thousand antennae, the number of setae ranged from 0 to 9, and an analysis of the distribution of the numbers showed that it could be interpreted as a normal probability distribution in which the number 4 occurs in excess, with a compensating deficiency of numbers greater than 4. This, together with the fact that 4 was also the number of setae removed, was made the basis of some theoretical discussion.

In the present work, most of the amputations have been performed not on young but on mature and therefore much larger animals, and the number of setae regenerated is substantially greater than in the earlier experiments, numbers above 4 being commoner than those below. (The maximum number, though in a very much smaller total than in the earlier experiments, is also 9.) It appears therefore that the number of setae regenerated is largely influenced by the area of the hypodermis which closes the wound and forms the new tip of the antenna from which the new setae are formed. Therefore the fact that operation in new-born animals tends to be followed by the reproduction of the missing number of setae has not all the significance that I attributed to it.

For operation, the animal is anaesthetized in a 5 per cent. solution of ether in water, and placed on a microscope slide to which a strip of celloidin has been cemented. The antenna is amputated by a splinter broken off from the edge of a safety-razor blade and mounted on a holder. The amputations were made near the distal ends of segments M or B on either the dorsal or ventral ramus, usually on the corresponding rami of both antennae. The loss of one ramus from both antennae causes no serious disturbance in the life of the animal, as judged by egg-production.

Except when there were reasons for the contrary, the operations were performed within an hour or two after ecdysis, in order to avoid the disturbing

factor of the presence, later in the instar, of the setae to be extruded at the next ecdysis. With operation early in an instar the sheath and core nuclei are removed, or if left in the antenna they disappear. The remains of the seta strand can often be identified for a while contracted to the base of the operated segment. In any case, neither the old seta cells if present, nor the seta strand, take any part in the production of new setae. These are formed entirely from the hypodermis which closes the wound.

The muscles cut through by the operation contract to the base of the operated segment and degenerate; about 4 or 5 days after operation they can still be identified as a nearly homogeneous mass. After two ecdyses after operation no remains of them can be identified in whole months. No formation of new muscles to replace the old ones was ever found. This, however, is not surprising, as the regenerating segment is now the terminal one, and this has no muscles even in the intact antenna.

The nerve disappears from the operated segment, and only once out of a large number of cases was a nerve found after formation of new setae is complete.

The whole process of regeneration concerns therefore only the formation of new setae.

Amputation is followed by an out-gush of blood, which clots to form a plug closing the cylinder of cuticle which is the outer wall of the antenna. After about 24 hours (at winter room temperatures, at which an adult instar lasts about 5 days) this plug has been transformed into a densely pigmented fibrous structure, containing cellular elements consisting doubtless of leucocytes and cells from the hypodermis, which becomes disorganized for a short distance below the wound.

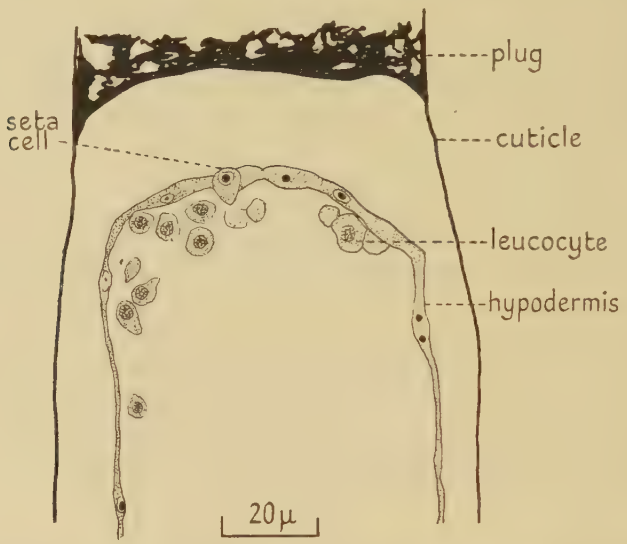
The hypodermis lining the cuticle has now grown across the wound below the plug, forming a thin membrane consisting of a single layer of flattened cells like the rest of the hypodermis.

About 40 hours after the operation the condition is as shown in Text-fig. 6. The reconstituted part of the hypodermis now forming the tip of the regenerating antenna, though still only one-layered, is becoming thicker than the rest of the hypodermis owing to the enlargement of its cells. In the figure, one cell in particular is seen to be extending below the inner boundary of the rest of the hypodermis. Examination of later stages shows that this is a future seta core cell. Its nucleus is already larger than those of typical hypodermal cells.

The number of leucocytes present in the injured segment is much greater than in the normal antenna. They are distributed throughout the segment, but are specially numerous close under the terminal hypodermal membrane from which the new setae are to be formed.

About 50 hours after operation there has been a general enlargement of the cells of the hypodermal membrane closing the wound. Some of them are sending out long processes into the lumen of the antenna; these are the future core cells. They become arranged in pairs, one still in the hypodermis, the other sunk below it. They soon, however, come to constitute a binucleate body in which a separating cell boundary cannot be distinguished.

Text-fig. 7 shows the condition after the lapse of one full instar, and therefore also of one ecdysis, after operation. (The smaller size of Text-fig. 7 compared with Text-fig. 6 is due to the fact that the former is a section through segment M, and the latter through segment B.) The cells of the hypodermis closing the wound have now enlarged enormously. On the left of the section a seta is seen growing out of a binucleate cell mass formed by two core cells. On each side of it is an elongating hypodermal cell, no doubt the future



Text-fig. 6. Section of the tip of an antenna 40 hours after amputation through segment B.

sheath cells. On the right can be seen a multicellular mass, cut obliquely; in neighbouring sections at least two setae can be seen to originate from this mass.

Like the primary embryonic setae, the regenerating seta at this stage is formed from the core cells only, and therefore corresponds to the distal segment of the adult seta.

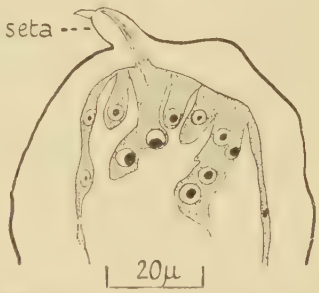
The ecdysis at the end of the instar in which the operation was performed carries away the plug at the end of the amputated segment, and the primary setae, which had grown out between the hypodermis and the plug, now project to the exterior.

During the instar initiated by this first ecdysis after operation (the instar the beginning of which is represented by Text-fig. 7) the full morphology of the seta apparatus is established. During this instar there is a rapid backgrowth of core and sheath cells to form the seta sac and strand, their advancing tips frayed out into pseudopodia-like processes reminiscent of a regenerating vertebrate nerve-fibre. When the operation was through segment M, by the following instar these processes had penetrated into segment B, where some of them came in contact with the muscle strands of that segment.

Whether this is a sufficient fixation basis for them, or whether they continue to grow back to their normal fixation point half-way down the protopodite, has not been ascertained.

At the second ecdysis after operation the sheaths are everted in the usual way, and the typical jointed setae, supplied with the two lines of hairs, appear. The setae are, however, still much shorter than the normal setae corresponding with the shorter seta sacs. They increase in length at subsequent ecdyses, but never reach the normal length in animals operated as adults.

The process of forming new setae after amputation is therefore similar to that in embryonic development; owing to the much larger area of the hypodermis involved, the number of setae regenerated is, however, often much larger than in embryonic development. I have not found any mitoses in the regeneration blastema, if one may so describe the area of hypodermis from which the new setae are formed, nor in the neighbouring parts of the hypodermis. The large mass of new protoplasm involved in the formation of half a dozen setae is provided entirely by the enlargement of individual cells.



TEXT-FIG. 7. Section of the tip of an antenna one full instar after amputation through segment M. The hypodermis has shrunk away from the cuticle in fixing.

As we have seen, the potentiality of seta-formation extends along the whole length of the dorsal and ventral rami, for new setae are formed whatever the level of amputation, even when this is through segment B of the dorsal ramus, which is far below the level of normal seta formation. The potentiality is not even confined to those cells which, as a consequence of the operation, have become constituents of the hypodermis forming the tip of the stump of the amputated antenna. Seta-formation can be induced by pricking an intact antenna anywhere along its length distal to the protopodite. This is followed by an enlargement of the hypodermal cells round the point of injury, which produce setae in the same way as described for the formation of new terminal setae. Seta-formation does not, however, result from a lateral wound so regularly as it does from the tip of an amputated antenna. Out of 37 operations in which a fine needle was passed through the wall of the antenna at various points along its length, 15 produced 1–7 setae each at the point of the wound. In the remaining 22, after throwing off the scar tissue at the next ecdysis, no setae appeared.

The protopodite, however, is either incapable of producing setae, or its capacity to do so is very low compared with that of the rami. Amputation through the protopodite proved too severe an operation, but sixteen protopodites were pricked, and scars formed over the wounds exactly similar in appearance to the scars formed by pricking the rami; not one seta, however, was produced.

Similar small wounds were made in the neighbourhood of the pair of abdominal setae, but no new setae were ever produced. The other parts of the body carrying setae of this type, the thoracic appendages, are not accessible to operation. Small wounds in various parts of the head and carapace heal rapidly, and, as was to be expected, without seta formation.

Thus any hypodermal cell of the antenna distal to the protopodite has the potentiality of becoming a seta-forming cell, given the necessary conditions. It is possible that one of these conditions is that the cell should be relieved from the tension of the thinly stretched hypodermis lining the antennar cuticle, thus giving it opportunity to enlarge. It will be noted that the setae formed in embryonic development are produced at the apices of segments, for even the lateral setae are terminal to the segments from which they spring.

In fact, we are here not concerned with regeneration in the sense of restoring the normal condition, but with the release of a potentiality which is normally inhibited owing to the lack of some further factor necessary for its realization. The problem of why certain cells at constant positions in the embryonic antenna develop setae, is equally the problem of why seta formation is restricted to these positions, since all the hypodermal cells distal to the protopodite are potential seta-producers.

It appears therefore that the determination of a seta cell takes place in two stages. First, there is the acquisition of a state of competence to differentiate into seta-forming cells. This occurs in certain regions of the body, namely, the antennae distal to the protopodite, the thoracic appendages, and a small region at the posterior bend of the abdomen. This competence is retained throughout life in the case of the rami of the antennae. The second stage in the determination is the local morphogenetic stimulus to which the response of a seta-formation is given. The situation is not greatly altered if for the provision of a positive morphogenetic stimulus we substitute the local removal of an inhibiting factor.

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The Blood-system in the Serpulimorpha (Annelida, Polychaeta)

II. The Anatomy of the Blood-system in the Sabellidae, and Comparison of Sabellidae and Serpulidae

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Summary

1. The blood-system in sabellids of the following genera is described: *Sabella*, *Potamilla*, *Branchiomma*, *Dasychone*, *Amphiglena*, *Fabricia*, *Jasmineira*, *Dialychone*, and *Myxicola*.

2. The central blood-system of *Sabella* is typical of the family, but the peripheral blood-system is variable.

3. The dorsal vessel lacks the valve and muscular sphincter found in some serpulids.

4. Lateral vessels are present only in *Sabella* and *Dasychone*.

5. The differences and similarities between sabellid and serpulid blood-systems are discussed. Special attention is given to the functions of sub-epidermal and coelomic capillaries and the blood-supply of the body-wall musculature.

Contents

Introduction

EWER (1941) has given a full account of the anatomy of the blood-system in *Sabella*. The present paper records observations on several other sabellids, and includes a discussion of the comparative anatomy of the blood-system in the Sabellidae. It concludes with a comparison of the blood-system of sabellids with that of serpulids, described in the first paper of this series (Hanson, 1950).

Previous Work

A general account of the anatomy of *Sabella* has been published by Thomas (1940). The body of a sabellid consists of a prostomium bearing a branchial crown, a thorax of several segments of which the first is the peristomium, and

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an abdomen. The branchial crown consists of filaments which bear pinnules. The anterior end of the thorax bears a collar.

The most detailed account of the blood-system of a sabellid is that of Ewer (1941), who has described *Sabella pavonina* and *S. spallanzanii*. A gut sinus and a ventral vessel, connected by a pair of ring vessels in each segment, extend from the posterior end of the body to the junction between stomach and oesophagus at the posterior end of the second thoracic segment. Here the sinus is continued forwards as two latero-dorsal vessels and a peri-oesophageal plexus. In the first segment (peristomium), the latero-dorsal vessels join to form a median dorsal vessel, which opens into a transverse vessel just behind the cerebral ganglia. From the transverse vessel lead two branchial vessels and two circum-oesophageal vessels, which join to form the ventral vessel. A pair of vessels from the peri-oesophageal plexus, and paired blind-ending vessels supplying the lateral lips, ventral sacs, and ventral collar folds all join the circum-oesophageal vessels in the first segment. A lateral vessel extends along each side of the body from the tip of the abdomen to the posterior end of the second thoracic segment, and is joined to each ring vessel by a lateral connective vessel. In each segment of both thorax and abdomen, the lateral vessel gives off a segmental dorsal vessel and a notopodial vessel; and the ring vessel has a trans-septal branch. All these branches end blindly. The segmental dorsal vessel lies on the coelomic surface of the dorsal longitudinal muscle block. The notopodial vessel supplies the capillaries in the coelomic pouch of the notopodium (notopodial 'gland'). The trans-septal vessel, after passing into the next posterior segment, divides into a neuropodial vessel, supplying the capillaries in the neuropodial gland, and a ventral gland-shield vessel. The lateral vessels, segmental dorsal vessels, and ventral gland-shield vessels give off numerous blind-ending capillaries projecting into the main peri-visceral coelom.

Ewer has reviewed the earlier work on *Sabella*. Other members of the sub-family Sabellinae were examined by Leydig (1851—*Amphiglena mediterranea*), Claparède (1868–70—*Dasychone lucullana*; 1873—*A. mediterranea*), Cosmovici (1879—*Branchiomma vesiculosum*), Brunotte (in Meyer, 1888—*B. vesiculosum*), Meyer (1888—*A. mediterranea*), de St.-Joseph (1894—*Bispira volutacornis*, *Dasychone bombyx*), Soulier (1903—*Branchiomma vesiculosum*), McIntosh (1918—*Bispira volutacornis*), Evenkamp (1931—*Laonome kroyeri*), and Fox (1938—*Dasychone bombyx*). Members of the Fabriciinae were studied by Schmidt (1848—*Fabricia sabella*), de Quatrefages (1850, 1865), Claparède (1862, 1873—*F. sabella*), Mecznikow (1865—*F. sabella*), Bourne (1883—*Manajunkia aestuarina*), Leidy (1883—*M. speciosa*), de St.-Joseph (1894—*Jasmineira elegans*), and Evenkamp (1931—*Euchone papillosa*). *Myxicola* was described by Claparède (1873—*M. infundibulum*), Cosmovici (1879—*M. infundibulum*), and Meyer (1888), and probably also by de Quatrefages (1865) whose *Amphicorina argus* was probably *M. aesthatica* (see Fauvel, 1927).

These earlier descriptions of sabellid blood-systems will be referred to in connexion with my own observations, and will be reviewed in the discussion

of the comparative anatomy of the blood-system (p. 374). The most important contributions are those of Claparède (1873), Meyer (1888), and Evenkamp (1931).

Material and Methods

The following species have been used: *Sabella pavonina* Savigny, *S. spallanzanii* (Viviani) (see Ewer, 1946), *Potamilla reniformis* (O. F. Müller), *P. stichophthalmos* (Grube), *Branchiomma vesiculosum* (Montagu), *Dasychone lucullana* (Delle Chiaje), *Amphiglena mediterranea* (Leydig), *Fabricia sabella* (Ehrenberg), *Jasmineira candela* (Grube), *Dialychone acustica* Claparède, *Myxicola infundibulum* (Rénier), and *M. aesthetica* (Claparède). *Sabella pavonina*, *Branchiomma*, and *Myxicola* were studied at Plymouth, and the other species at Naples.

Many of the observations on the smaller species were made on whole living specimens. Dissections of the larger species were made under a binocular dissecting microscope. The methods used in the preparation of serial sections have been recorded in the previous paper of this series (Hanson, 1950).

Observations

1. *Sabella spallanzanii* and *S. pavonina*. In dissections of the abdomen of living specimens I have confirmed Ewer's account (1941) of the arrangement of the following vessels: gut sinus, ventral vessel, lateral vessels, ring vessels, lateral connective vessels, segmental dorsal vessels, notopodial vessels, transseptal vessels, ventral gland-shield vessels, neuropodial vessels. In serial sections through the anterior part of the thorax of *S. spallanzanii*, I have confirmed Ewer's description of the arrangement of the latero-dorsal, dorsal, transverse, circum-oesophageal, and branchial vessels. The dorsal vessel lacks both the valve and the muscular sphincter found in many serpulids (Hanson, 1950). Although Ewer has stated that the thoracic nephridia have no blood-supply, I have noticed numerous small vessels lying in their walls, but have not been able to trace them to their source.

2. *Potamilla reniformis* and *P. stichophthalmos*. In serial sections through a species of *Potamilla* (either *P. reniformis* or *P. stichophthalmos*) the gut sinus, latero-dorsal vessels, dorsal vessel, transverse vessel, branchial vessels, circum-oesophageal vessels, ventral vessel, and ring vessels have the same arrangement as in *Sabella*. A peri-oesophageal plexus, communicating posteriorly with the gut sinus, is situated in the first two thoracic segments. No connexions with the circum-oesophageal vessels could be found. There was no evidence that the plexus is double as it is in some serpulids (Hanson, 1950). A collar vessel branches off the circum-oesophageal vessel on each side near the posterior end of the first segment. The dorsal vessel lacks the valve and muscular sphincter found in some serpulids. At the base of each branchial vessel the muscle coat is thickened to form a sphincter. There are no blind-ending capillaries projecting into the coelom. No lateral vessels could be found.

In three living specimens of *P. stichophthalmos*, the gut sinus, ventral vessel, ring vessels, latero-dorsal vessels, and median dorsal vessel were visible. These specimens were mature females. The anterior part of the abdomen contained no reproductive cells; but the coelom of the posterior part was full of ova, and the body-wall muscles were apparently all broken down. (Prenant (1929) has found that sarcolysis proceeds farther in *Potamilla* than in other sabellids and serpulids.) In this genital part of the abdomen the blood-system is more distinct than in the anterior part, because of the attenuated body-wall. Latero-dorsally, the ring vessels appear to be detached from the septa; they are long and looped. Ventrally, each ring vessel gives off a vessel which traverses the septum and extends along the next posterior segment, ending blindly near the ring vessels. On its course it gives off short, fat, blind-ending capillaries. These trans-septal vessels could not be distinguished in the anterior region of the abdomen. Here, the ring vessels apparently adhere to the septum throughout their course. No lateral vessels could be found in either part of the abdomen. There were no blind-ending capillaries projecting into the coelom.

In living specimens of *P. reniformis*, the gut sinus, ventral vessel, latero-dorsal vessels, median dorsal vessel, and the ring vessels in the posterior part of the abdomen were like those of *P. stichophthalmos*. No lateral vessels or coelomic capillaries could be found.

3. *Branchiomma vesiculosum.* A few living specimens were dissected, and the following observations were made on the abdominal blood-system. The ventral vessel, gut sinus, and ring vessels are present. A portion of each ring vessel, detached from the septum, projects into the mouth of the segmental organ. This part of the ring vessel gives off a large tuft of blind-ending capillaries which project into the main perivisceral coelom. These are the only coelomic capillaries; but several strands of tissue, containing chloragosomes and many capillaries, stretch across the coelom from near the mid-ventral line to the latero-dorsal part of the body-wall. These strands receive their blood-supply from small branches of the ring vessels. Each ring vessel also gives off several branches to the intersegmental septum and several other branches leading to the capillaries on the coelomic surface of the dorsal longitudinal muscle block. The surface of the ventral muscle block apparently lacks a blood-supply. No trans-septal vessels and no lateral vessels could be found. Soulier (1903) described lateral vessels in *B. vesiculosum*, but Brunotte (in Meyer, 1888) could not find them.

4. *Dasychone lucullana.* In living specimens the only vessels that are clearly distinguishable are the conspicuous lateral vessels of the abdomen, and their branches. In each segment the lateral vessels have blind-ending branches which appear to correspond to the segmental dorsal vessels and the notopodial vessels of *Sabella*. The lateral vessels bear numerous blind-ending capillaries projecting into the coelom. In a sectioned specimen it was found that the latero-dorsal, dorsal, transverse, and circum-oesophageal vessels, and the peri-oesophageal plexus, have the same arrangement as in *Potamilla* and *Sabella*.

5. *Amphiglena mediterranea.* In living specimens it can be seen that a gut sinus, ventral vessel, and ring vessels are present. The sinus ends anteriorly at the boundary between the second and third thoracic segments, as already recorded by Meyer (1888). In the abdomen, but not in the thorax, each ring vessel gives off an unbranched trans-septal vessel, which ends blindly in the parapodium of the next posterior segment; it resembles the trans-septal vessel in the small serpulids *Salmacina* and *Spirorbis* (Hanson, 1950). Lateral vessels appear to be absent. I cannot confirm Meyer's statement that they are present in the abdomen; possibly he mistook for them the trans-septal vessels. No blind-ending coelomic capillaries are present.

6. *Fabricia sabella.* In living specimens the gut sinus, ventral vessel, and ring vessels were found. The ring vessels appeared to lack branches. No lateral vessels and no coelomic capillaries were present.

7. *Jasmineira candela.* In living specimens the gut sinus, ventral vessel, and ring vessels were found. Each ring vessel, in both thorax and abdomen, gives off a trans-septal vessel ending blindly in the parapodium of the next posterior segment. These trans-septal vessels bear blind-ending capillaries projecting into the coelom. No other coelomic capillaries and no lateral vessels could be found.

8. *Dialychone acustica.* One living specimen was examined. Only the abdominal blood-system could be distinguished clearly. It closely resembled the blood-system of the posterior part of the abdomen of *Potamilla stichophthalmos*, described above.

9. *Myxicola infundibulum.* The main vessels of the abdomen and the posterior segments of the thorax have been investigated in dissections of living specimens. In each segment a pair of ring vessels connects the ventral vessel with the gut sinus. Laterally, each ring vessel gives off two branches, both of which pass backwards through the intersegmental septum and enter the next segment. The more dorsal vessel, which I shall call the dorsal trans-septal vessel, branches into numerous capillaries lying on the coelomic surface of the dorsal longitudinal muscle block. When the muscle block is split open one can see many capillaries passing straight through it from the coelomic surface to the outer surface. Transverse sections of fixed specimens show that numerous capillaries lie on the outer surface of the muscles, just under the thick glandular epidermis. The pigment in the epidermis obscures these capillaries in living specimens. The other branch of the ring vessel is the ventral trans-septal vessel which passes towards the surface of the body. I have been unable to trace it far from the septum. It may perhaps supply the capillaries lying under the ventral epidermis. This ventral trans-septal vessel gives off two branches almost immediately after it has left the ring vessel. One of these vessels passes through the septum and branches into numerous capillaries lying on the posterior surface of the septum. The other vessel does not traverse the septum, but branches into numerous capillaries lying on the coelomic surface of the ventral longitudinal muscle block. Unlike the branches of the dorsal trans-septal vessel these capillaries apparently do not enter the muscle block.

The dorsal trans-septal vessel and the vessel supplying the septum were noticed by Claparède (1873), who stated that they were branches of the lateral vessel. I have been unable to find any lateral vessels. There are no capillaries projecting into the coelom.

In a series of sections through the anterior part of the thorax of one specimen the main blood-vessels have been traced. The gut sinus leads anteriorly into a peri-oesophageal plexus and into two latero-dorsal vessels, which farther forward join to form a single median dorsal vessel. The latter enters a transverse vessel which at each end forks into a branchial vessel and a circum-oesophageal vessel. The circum-oesophageal vessels meet ventrally to form the ventral vessel. Each of them gives off several branches to the nephridium, which has numerous vessels in its walls. I could find no connexions between the peri-oesophageal plexus and the circum-oesophageal vessels. There is no valve in the dorsal vessel and it has no muscular sphincter.

10. *Myxicola aesthetica.* The blood-system has been studied only in intact living specimens: *M. aesthetica* is small and fairly transparent. In each segment behind the second thoracic segment the gut sinus and ventral vessel are connected by two ring vessels, each of which has a single branch. This branch passes backwards through the intersegmental septum into the next segment, where it divides into three vessels. One of them branches into capillaries in the septum. The other two vessels, dorsal and ventral, branch into capillaries on the coelomic surfaces of the dorsal and ventral longitudinal muscle blocks. No lateral vessels and no coelomic capillaries could be seen. The gut sinus and the ventral vessel end anteriorly at the junction of the second and third thoracic segments. The sinus leads into two latero-dorsal vessels, which meet farther forward to form a single median dorsal vessel. This enters a transverse vessel from which lead the branchial and circum-oesophageal vessels.

Discussion

It is now possible to discuss which are the features common to the blood-systems of all sabellids, and in which ways the genera differ from each other.

A gut sinus and ventral vessel communicating by segmentally arranged ring vessels are known to occur in *Sabella*, *Potamilla*, *Branchiomma*, *Dasychone*, *Amphiglena*, *Fabricia*, *Jasmineira*, *Dialychone*, and *Myxicola*, and in *Laonome* and *Euchone* (Evenkamp, 1931). In *Sabella*, *Potamilla*, *Dasychone*, *Myxicola*, *Laonome*, and *Euchone* the sinus communicates with the ventral vessel at the anterior end of the body by way of latero-dorsal, dorsal, transverse, and circum-oesophageal vessels situated in the peristomial and second thoracic segments. The latero-dorsal vessels are in the second segment, the median dorsal and transverse vessels in the peristomium; the circum-oesophageal vessels extend through both segments. *Amphiglena* is perhaps similar: Meyer (1888) has described dorsal and circum-oesophageal vessels, both extending through the first two segments, but future observations may show that the posterior part of the dorsal vessel is paired. A median dorsal vessel

has briefly been recorded in *Bispira* (McIntosh, 1918), *Fabricia* (Claparède, 1862; Mecznikow, 1865), *Oridia* (de Quatrefages, 1865), and *Jasmineira* (de St.-Joseph, 1894). Soulier's account (1903) of the anterior blood-system of *Branchiomma* supports Claparède's later view (1873) that dorsal and circum-oesophageal vessels are absent in sabellids, their place being taken by a peri-oesophageal plexus. Ewer (1941) and others have shown that Claparède was mistaken.

Thus in all species which have been examined sufficiently carefully the central blood-system is like that of *Sabella* as described by Ewer (1941).

The dorsal vessels of *Sabella*, *Potamilla*, *Dasychone*, and *Myxicola* lack the valve and muscular sphincter found in some serpulids (Hanson, 1950). Other sabellids have not yet been examined for these structures.

Sabella, *Potamilla*, *Dasychone*, and *Myxicola* possess a peri-oesophageal plexus extending through the first two thoracic segments and opening out of the gut sinus. In *Sabella* Ewer (1941) has described connexions with the circum-oesophageal vessels. The presence of a peri-oesophageal plexus in *Branchiomma* has been recorded by Soulier (1903) but not adequately confirmed.

The blood-supply of the body-wall and parapodia shows wide variations within the family. Lateral vessels are certainly present in *Sabella* and *Dasychone*. They have also been described in *Laonome* (Evenkamp, 1931). I have been unable to confirm previous accounts of their presence in *Branchiomma*, *Amphiglena*, and *Myxicola*, and I could not find them in *Potamilla*, *Fabricia*, *Jasmineira*, and *Dialychone*. It is probable that previous observers mistook trans-septal vessels for lateral vessels. In *Sabella* and *Dasychone* the blood-supply of the body-wall and parapodia is derived partly from the lateral vessels, partly from the ring vessels. In other genera which have been adequately studied the body-wall and parapodia are supplied only by branches of the ring vessels. In some cases these branches supply the segment in which they originate; in other cases they pass backwards into the next segment before branching. The distribution of these vessels varies greatly from species to species.

Blindly ending capillaries, projecting into the perivisceral coelom, are present in *Sabella*, *Branchiomma*, *Dasychone*, and *Jasmineira*, and are known to be absent in *Potamilla*, *Amphiglena*, *Fabricia*, *Dialychone*, and *Myxicola*. Their functions will be discussed later.

Comparison of Sabellidae and Serpulidae

Although the largest members of the Serpulimorpha belong to the Sabellidae, there is a wide variation in size within each family. The smallest sabellids are no larger than the smallest serpulids, and not many sabellids are larger than the serpulid *Protula intestinum*. In the first paper of this series (Hanson, 1950) I have dealt with the blood-system in the Serpulidae. I shall now discuss the similarities and differences between the blood-systems of the various serpulids and sabellids which have so far been investigated. I shall show that

some of them are fundamental differences between the two families whilst others are to some extent correlated with body size.

The blood-system in the Serpulimorpha consists of a central blood-system through which a continuous true circulation of blood is probably maintained, and of a peripheral blood-system of predominantly blind-ending vessels which are alternately empty and full, receiving their blood from the central system, then returning it along the same channels to the central system.

The central blood-system is built on the same plan in both families. The blood moves forwards in a sinus surrounding the alimentary canal, and backwards down a ventral vessel. The ventral vessel and the sinus communicate with each other by segmentally arranged ring vessels, and by a short dorsal vessel, a transverse vessel, and a pair of circum-oesophageal vessels situated at the anterior end of the thorax.

The two families differ in the arrangement of the vessels at the anterior end. The junction of stomach and oesophagus is situated at the boundary of the second and third thoracic segments in the Sabellidae, and of the first and second segments in the Serpulidae. This difference is reflected in the blood-systems. In the Serpulidae, the sinus and ventral vessel terminate anteriorly at the front of the second segment, and the dorsal, transverse, and circum-oesophageal vessels are situated entirely within the first (peristomial) segment. In the Sabellidae the sinus and ventral vessel terminate at the front of the *third* segment. The circum-oesophageal vessels extend through the first two segments. The transverse vessel is in the same position as in the Serpulidae. The dorsal vessel consists of a single median vessel in the peristomium, and is represented by two latero-dorsal vessels in the second segment.

The dorsal vessel in some of the larger serpulids possesses a valve and a muscular sphincter, probably preventing back-flow of blood from the transverse vessel into the dorsal vessel. None of the sabellids so far studied in sufficient detail possess the valve or sphincter. Ewer (1941) has discussed the control of blood-flow at the anterior end of *Sabella*.

The peripheral blood-system has the following components, which will be discussed in this order: the branchial vessels and their branches; the peri-oesophageal vascular plexuses; the vessels of the collar and lips; the vessels supplying the body-wall and parapodia.

The single vessels in each filament, pinnule, and 'palp' of the branchial crown, and the vessels of serpulid opercula, are all branches of two branchial vessels, which leave the central blood-system at the junctions of the circum-oesophageal vessels with the transverse vessel.

A peri-oesophageal plexus opening out of the gut sinus has been found in the larger serpulids and larger sabellids. It is absent in the smallest serpulids. It is not yet known if the plexus is also absent in the smallest sabellids. Some serpulids possess two independent plexuses, one communicating with the gut sinus, and the other with the circum-oesophageal vessels. Two plexuses have not yet been found in any sabellid. In *Sabella* Ewer (1941) has found a single

plexus communicating posteriorly with the sinus and anteriorly with the circum-oesophageal vessels. In sabellids the plexus extends through the first two segments; in serpulids it is confined to the first segment.

In both families the vessels of the collar and lips are typically derived from branches of the circum-oesophageal vessels.

The blood-supply of the body-wall and parapodia of serpulids and sabellids is derived from ring vessels and lateral vessels. The latter, where present, extend along each side of the body and communicate with each ring vessel. Lateral vessels are absent in all the serpulids which have been examined. Among the Sabellidae, only *Sabella* and *Dasychone* are definitely known to possess them. In the Serpulidae the body-wall and parapodia of each segment are supplied with blood by trans-septal vessels, which are branches of the ring vessels of the preceding segment. The Sabellidae show much greater variation in the arrangement of the vessels supplying the body-wall and parapodia. They may be branches of ring vessels or of lateral vessels; they may supply either the segment in which they originate, or the next posterior segment.

The well-developed longitudinal muscles of the body-wall of serpulids and sabellids lack a special blood-supply. In the smallest species of both families no vessels pass through the muscle blocks. In the larger serpulids, and in *Myxicola infundibulum*, some branches of the dorsal trans-septal vessels penetrate the dorsal muscle blocks but do not branch in them. Ewer (1941) has found no vessels in the longitudinal muscle blocks of *Sabella pavonina*, but a few vessels are present in the ventral blocks of *S. spallanzanii* and in both dorsal and ventral blocks of *Branchiomma*. It is clear, however, that oxygen must reach the muscles mainly by diffusion from either the outer or the inner (coelomic) surfaces. In the smallest species this presents no special difficulties. In the larger species it seems necessary for one or both surfaces to possess a rich supply of blood-vessels, or to be in contact with a circulating fluid in which the oxygen pressure is maintained at a high level.

The body-surface in the larger serpulids has a rich blood-supply, and the water in contact with this surface is constantly renewed. It seems probable that the body-surface of these serpulids is respiratory and supplies oxygen to the muscles lying underneath. In the largest serpulid, *Protula intestinum*, not only have the outer surfaces of the muscle blocks a rich blood-supply, but the coelomic surfaces, also, are covered by numerous capillaries. A similar rich blood-supply to the coelomic surfaces of the muscles is also found in the large sabellids *Branchiomma* and *Myxicola infundibulum*. In *Sabella* this is not the case. As Fox (1938) has suggested, the numerous blind-ending capillaries which project into the coelom of *Sabella* may aerate the coelomic fluid and thus indirectly increase the oxygen-supply to the muscles. Coelomic capillaries are also present, but in much smaller numbers, in *Branchiomma* and in *Dasychone*. Numerous capillaries are situated on the outer surfaces of the muscle blocks of *Myxicola*, but they are probably of much more importance in supplying the glandular epithelium overlying them than the muscles.

In *Sabella* the body-surface, excepting the ventral gland-shields, is poorly supplied with capillaries.

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The Autonomic Nervous System of the Chimaeroid Fish *Hydrolagus colliei*

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With two Plates

Summary

The autonomic nervous system of the chimaeroid fish *Hydrolagus colliei* has been investigated by dissections and histological methods. It consists of a cranial parasympathetic portion and a sympathetic portion confined to the trunk. The latter extends from the level of the heart to the anus and consists of segmentally arranged ganglia on each side of the dorsal aorta. These ganglia are closely associated with small accumulations of suprarenal tissue. Two axillary bodies are the largest of the sympathetic and suprarenal structures. They lie about the subclavian arteries and are made up of a gastric ganglion and a relatively large mass of chromaffin tissue. The sympathetic ganglia lie in an irregular plexus of longitudinal and crossing sympathetic strands but there is no regular sympathetic chain or commissure between ganglia. There are white rami communicantes which connect the sympathetic ganglia with spinal nerves. A small pregastric ganglion lies on the rami communicantes to the gastric ganglion. The visceral nerves arising from the sympathetic ganglia proceed to blood-vessels, genital ducts, chromaffin tissue, and gut. The latter is supplied by large splanchnic nerves which originate in the gastric ganglia and proceed along the coeliac axis to the intestine, pancreas, and liver. Prevertebral ganglia are absent. A mucosal and a submucosal plexus are present in the intestine. The cranial component of the autonomic system comprises a midbrain and a hindbrain outflow. In the former there is a ciliary ganglion on the inferior oblique branch of the oculomotor nerve. Short ciliary nerves proceed from this branch to the eyeball. A radix longa is absent. Sensory fibres go directly to the eyeball from the profundus nerve as anterior and posterior long ciliary nerves. The hindbrain outflow comprises scattered nerve-cells and ganglia on post-trematic branches of the glossopharyngeal and vagus nerves. These autonomic fibres in the branchial nerves innervate smooth muscle in the pharyngeal region. A visceral branch of the vagus innervates the heart, oesophagus, and intestine; it also establishes a connexion with the pregastric ganglion. In general, the autonomic nervous system of *Hydrolagus* is very similar to that of selachians. It appears that the autonomic systems of these two groups have undergone little alteration since their origin in the Palaeozoic from some common form. Their autonomic systems reflect a simple and primitive level of organization from which more complex systems of the bony fishes and amphibians have evolved.

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Contents

Introduction

THE autonomic nervous system of selachians (sharks and rays) has been studied by several workers and detailed accounts are available for this group. In organization and function it shows a number of peculiar features of a primitive nature when compared with higher vertebrates. The selachians themselves are a primitive group and the arrangement of their autonomic system represents a simple level of organization from which the more complex systems of teleostomes and tetrapods could have evolved. There is another and quite distinct group of elasmobranchs, however, in which the autonomic system is very imperfectly known: these are the chimaeroid fishes or Holocephali. Since a representative of this group is common locally in British Columbian waters, namely the ratfish, *Hydrolagus colliei*, an investigation of its autonomic nervous system has been initiated. The object of this study has been to obtain sufficient data to determine the morphological organization of its autonomic nervous system. With this pattern established comparisons will be made with the autonomic systems of other recent elasmobranchs, and the evidence obtained will be discussed in terms of the phylogeny of the system.

Owing to unforeseen circumstances it has not been possible to pursue this investigation so as to reach all objectives that were set. However, it is believed that the information obtained is sufficient to warrant publication in its present form, and that it represents a useful whole.

Only a few authors have referred to the autonomic system of chimaeroid fishes. Leydig (1851, 1853) showed that the so-called axillary hearts on the subclavian arteries of *Chimaera* and selachians are not contractile structures, but comprise suprarenal tissue comparable to the adrenal medulla of mammals. Additional segmentally arranged suprarenal bodies occur on the segmental arteries and are closely related to the sympathetic ganglia. Chevrel (1887) had a single alcohol-preserved specimen available for examination. He stated that the sympathetic system resembles that of dogfishes and rays. The first suprarenal body is situated on the subclavian artery and is followed by 12 to 14 similar bodies which continue to the posterior end of the abdomen.

Chevrel also distinguished several visceral nerves. However, no details are available for sympathetic ganglia, connectives, distribution of visceral nerves, and relations with the parasympathetic system. Finally, Schwalbe (1879) and Cole (1896) have described the ciliary ganglion of *Chimaera monstrosa*.

Material and Methods

Specimens of *Hydrolagus colliei* (Lay and Bennett) were obtained from shrimp trawlers operating in the Gulf of Georgia in the vicinity of Vancouver harbour. It was only occasionally possible to obtain specimens of fish alive since they live for only a few hours after capture, even when placed in aerated sea-water. Specimens and tissues were preserved either at the time of capture, or soon after the return of the boat. Fresh specimens and formalin-preserved specimens were dissected and the course of nerves followed either by naked eye or under a binocular microscope. In order to facilitate observation the nerves were blackened by the application of a solution of osmium tetroxide.

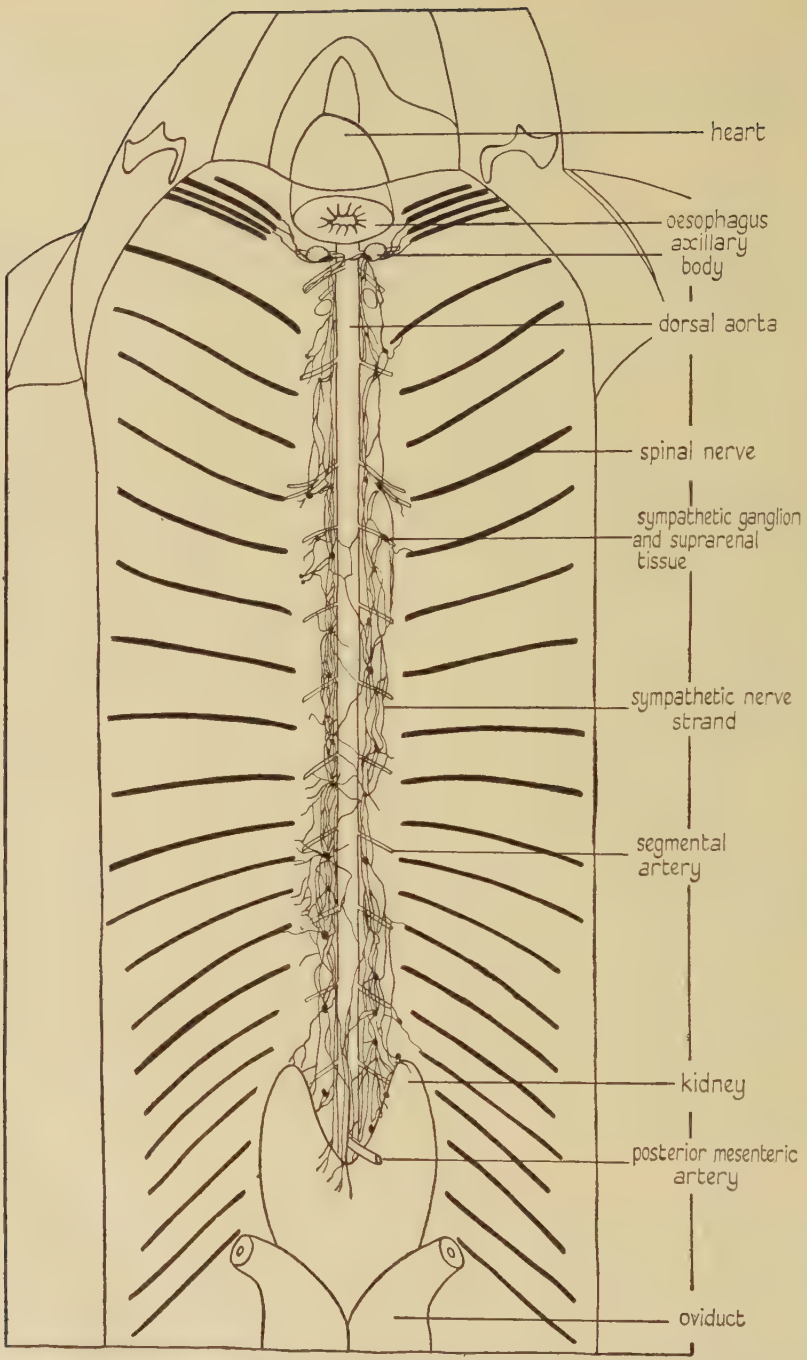
For the study of microscopic anatomy and histology, material was fixed as a routine in 10 per cent. formol and in Bouin's fluid. Two determinations of freezing-point depression of whole blood (mean $\Delta = 1{\cdot}49$) and a study of haemolysis in saline solutions of different concentrations indicated that the blood of this species has an osmotic pressure equivalent to a 2·6 or 2·7 per cent. solution of NaCl. After these determinations 27 gm. of NaCl were added to each litre of fixing fluids whenever possible to improve cellular preservation (Young, 1933*a*).

Sections were stained with Harris's haematoxylin and eosin, toluidine blue and safranin, or impregnated with silver. Two silver-on-the-slide methods were used with success and gave similar results, namely, Bodian's activated protargol and Holmes's buffered silver-pyridine method (Holmes, 1947). In preparing the incubating solution for Holmes's method, a pH of 8·4 and a silver concentration of 1/50,000 were used. Greater differentiation between nervous and connective tissues was obtained with formol-fixed material. After Bouin-fixation all fibrous and cellular elements tended to be equally darkened. Serial sections of specimens about 5 in. long were especially valuable for establishing details of micro-anatomy. These are later referred to as small specimens. In addition, some frozen sections were treated by the Gros-Bielschowsky method.

Observations

Sympathetic Ganglia and Cells

Sympathetic ganglia are confined to the trunk and form a bilaterally arranged series extending from the subclavian artery to the level of the anus. Generally there are two ganglia to each spinal segment but additional ganglia are often present (Text-fig. 1). The ganglia are intimately related to the suprarenal bodies, and are found near the latter structures or fused with them. The first ganglia of this series are the two 'gastric' ganglia which lie ventral to the



TEXT-FIG. 1. Outline drawing of the sympathetic nervous system of the ratfish. Dissection exposing the dorsal body wall of an adult ♀, × about 1·1.

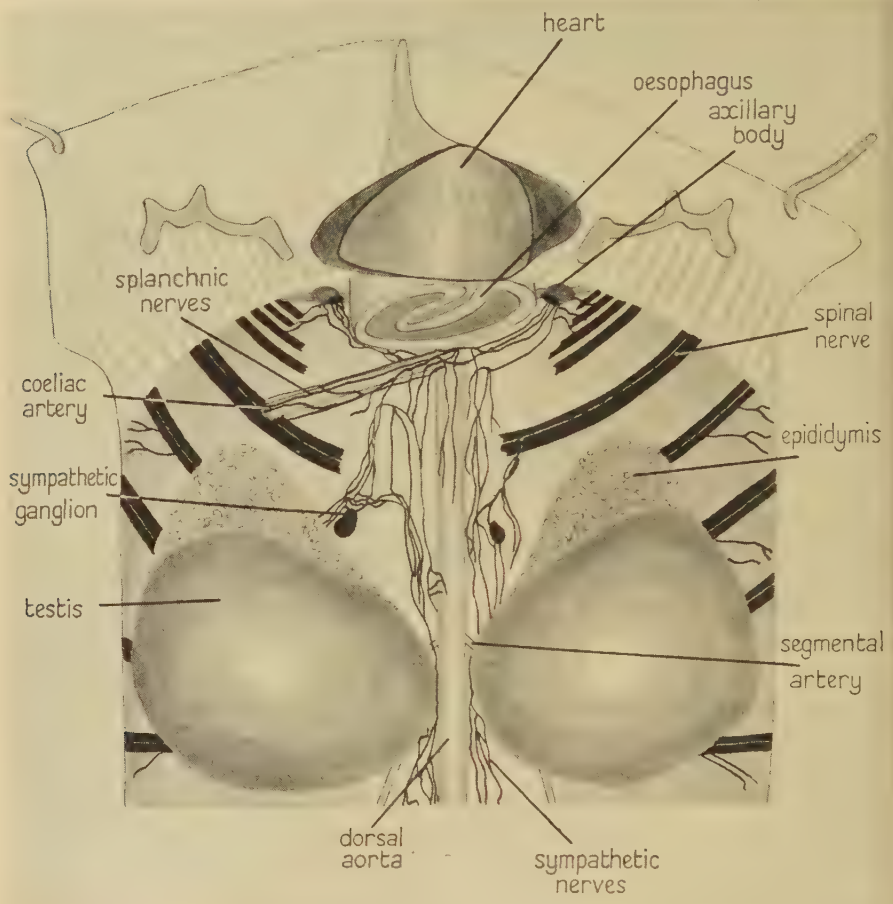
subclavian arteries near their origin from the dorsal aorta. These ganglia are closely associated with the first suprarenal bodies, and the whole complex of gastric ganglion and suprarenal body is termed the axillary body (Pl. I, fig. 1). This complex is organized in a similar manner to, and is homologous with, the axillary body of selachians (Chevrel, 1887; Leydig, 1851, 1852; Young, 1933*a*). In selachians, the axillary body supplies visceral nerves to the anterior viscera, including the stomach. In chimaeroids, however, a stomach is wanting. The absence of a stomach is probably a secondary feature, and the term 'gastric' ganglion is retained for reasons of homology and convenience.

The gastric ganglion lies in the wall of the posterior cardinal sinus. Posterior to this level the details differ somewhat for the two sexes but the fundamental pattern is similar. The mesonephros extends far forwards in the male. Between the level of the gastric ganglion and the mesonephros there are about three small sympathetic ganglia on each side of the median axis (Text-fig. 2). In the female the mesonephros lies more posteriorly and there are many more sympathetic ganglia in the interval between the gastric ganglia and the kidney (Text-fig. 1). They lie in the dorsal wall of the posterior cardinal sinuses, near the outer angle of the haemal ridges of the vertebrae. The ganglion cells are situated in discrete groups or they are conjoined to the suprarenal bodies. The segmental arrangement of anterior sympathetic ganglia and suprarenals is revealed by their association with segmental arteries proceeding to the body-wall.

In the kidney region the sympathetic ganglia and suprarenals shift ventrally and come to lie in the inferior wall of the posterior cardinal veins above each kidney (Pl. I, figs. 3, 4). The ganglia maintain their segmental arrangement, one or several ganglia corresponding to each spinal nerve. Renal ganglia and suprarenals tend to be larger than more anterior sympathetic ganglia, with the exception of the axillary body. They continue to the posterior abdominal region and stop at the level of the anus. No ganglion occurs in the small post-anal mesonephros which extends into the beginning of the tail, or in the haemal canal. In the posterior kidney region the ganglia become very small.

The arrangement of ganglionic and suprarenal tissues in *Hydrolagus* is very similar to that found in selachians. Chevrel (1887) distinguished two groups of ganglia, one group lying in front of the kidney, and a second group lying above the kidney. The latter group is also characterized by its more segmental arrangement according to this author. Young (1933*a*), however, has shown that such a distinction is a very difficult one to maintain, the ganglia of both groups forming part of one continuous segmental series. The same arrangement obtains in *Hydrolagus* where all the ganglia constitute a homogeneous system. *Hydrolagus* also agrees with other elasmobranchs in the absence of a caudal sympathetic system. Ganglia, longitudinal cords, and ganglion cells are definitely absent from the haemal canal both of adults and of the smallest specimens that we have been able to obtain (5 in. in length). Hoffmann (1900) and Young (1933*a*) identified caudal sympathetic ganglia in embryos of *Scyllium*, *Squalus*, and *Torpedo*, but they disappear in the adult.

The sympathetic ganglia frequently bulge into the cavity of the posterior cardinal veins. In the kidney region the ganglion cells lie in the median portion of the suprarenal mass, or occur as discrete ganglia separate from the suprarenal. They are also closely related to the segmental and renal arteries

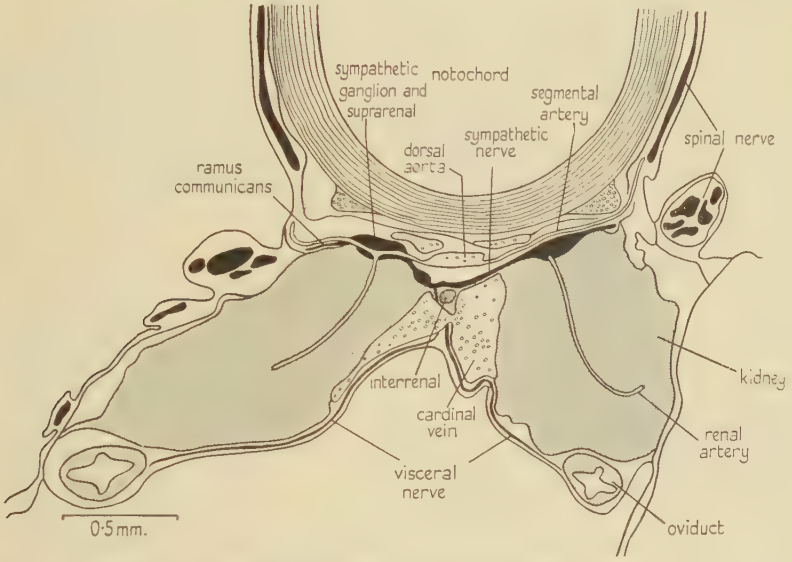


TEXT-FIG. 2. Drawing of dissection of adult ♂ ratfish. Anterior abdominal region. × 1·5.

(Text-fig. 3; Pl. I, figs. 2, 4). The renal arteries arise independently from the dorsal aorta, or as ventral branches of the segmental arteries. Ganglia and suprarenal bodies either (*a*) lie on the ventral face of a segmental artery as it proceeds laterally above the kidney, or (*b*) envelop a segmental artery, or (*c*) envelop a renal artery where it descends into the kidney tissue. Rarely, portions of ganglia and suprarenals actually accompany the renal artery into the mesonephros, where they are surrounded by kidney tubules. In addition to the main sympathetic ganglia, small groups of nerve-cells occur beside the

dorsal aorta. These groups are connected with the ganglia by small sympathetic nerves (Text-fig. 3). Young (1933*a*) has figured a similar arrangement in selachians, where one or several ganglia lie median and ventral to the segmental ganglia on the proximal course of the visceral nerves. These secondary ganglia are rather infrequent in *Hydrolagus* and may be regarded as fortuitously segregated components of the main ganglia.

The gastric ganglia are preceded by several diffuse 'pregastric' ganglia. The pregastric ganglia occur on the course of the rami communicantes leading



Text-fig. 3. Drawing of a transverse section through the kidney region of a young ratfish. Camera lucida from one section; details added from neighbouring sections.

from anterior spinal nerves to the gastric ganglia. They may be found in the superficial dorsal wall of the oesophagus, just beneath the posterior cardinal sinus. Each visceral vagal nerve, on entering the oesophageal wall, also sends a short branch dorsally to the ipsilateral pregastric ganglion. Similarly arranged pregastric ganglia and vagal connexions have been described in other elasmobranchs. In *Scyllium*, Chevrel (1887) observed several small ganglia on the rami communicantes supplying the gastric ganglion, and Young (1933*a*) found that there is often a small ganglion on the course of the anterior rami communicantes at the front end of the cardinal sinus. Both these authors have also described a connexion between the gastric ganglia and the visceral vagus in selachians. Chevrel claimed that sympathetic fibres proceed anteriorly into the oesophagus to reach the vagus nerve. Young found a similar connexion in some specimens of *Scyllium*, but regarded it as a contribution of preganglionic fibres from the vagus to the gastric ganglion. In *Hydrolagus* the connexion of the pregastric ganglia with the vagus is comparable to the similar junction between sympathetic and parasympathetic in selachians.

The exact relationship is uncertain: it may represent a vagal contribution o pre-ganglionic fibres in the nature of a ramus communicans to the pregastri and gastric ganglia, but the possibility cannot be excluded that these vaga fibres are discrete and run through sympathetic pathways to the end-organs.

A detailed cytological study of the sympathetic nerve-cells was not completed, but within certain limits it was observable that these cells in the ratfish display the characters typical of such neurones. All ganglionic neurocytons

TEXT-FIG. 4. Sympathetic nerve-cells in the gastric ganglia of adult ratfish. *a*, *c*, some arrangements of nerve-fibres; *b*, unusual form of sympathetic cell. *a*, Gros-Bielschowsky; *b* and *c*, Holmes's method.

are encapsulated and surrounded by satellite cells (Text-fig. 4). They are about 35μ in diameter (in the adult animal), but occasionally they are very large and of bizarre shape (Text-fig. 4*b*). They are usually uninucleate, but binucleate cells are frequent, and some are even multinucleate; one or two large nucleoli are present. Multipolar cells can be observed with one large axonal process; possibly all cells are of this category. Within the ganglia extra-capsular dendrites and pre-ganglionic fibres ramify in an intricate network among the nerve-cells. Large pre-ganglionic fibres appear to be wrapped about the cell bodies, and other fine fibres—subcapsular dendrites—encircle their cells. The picture is one of considerable complexity, but there are obvious opportunities for contact and trans-synaptic transmission, without invoking a neurosyncytial hypothesis (cf. Nonidez, 1944). Young (1933) has described and figured the autonomic neurocytons of selachians in greater detail. Besides encircling whirls of dendrites and axons, he found dense extra-

capsular glomeruli permitting association between pre- and post-ganglionic fibres, and extra-capsular boutons on amphicytes. Diamare (1901) also has described multinuclear sympathetic nerve-cells in selachians.

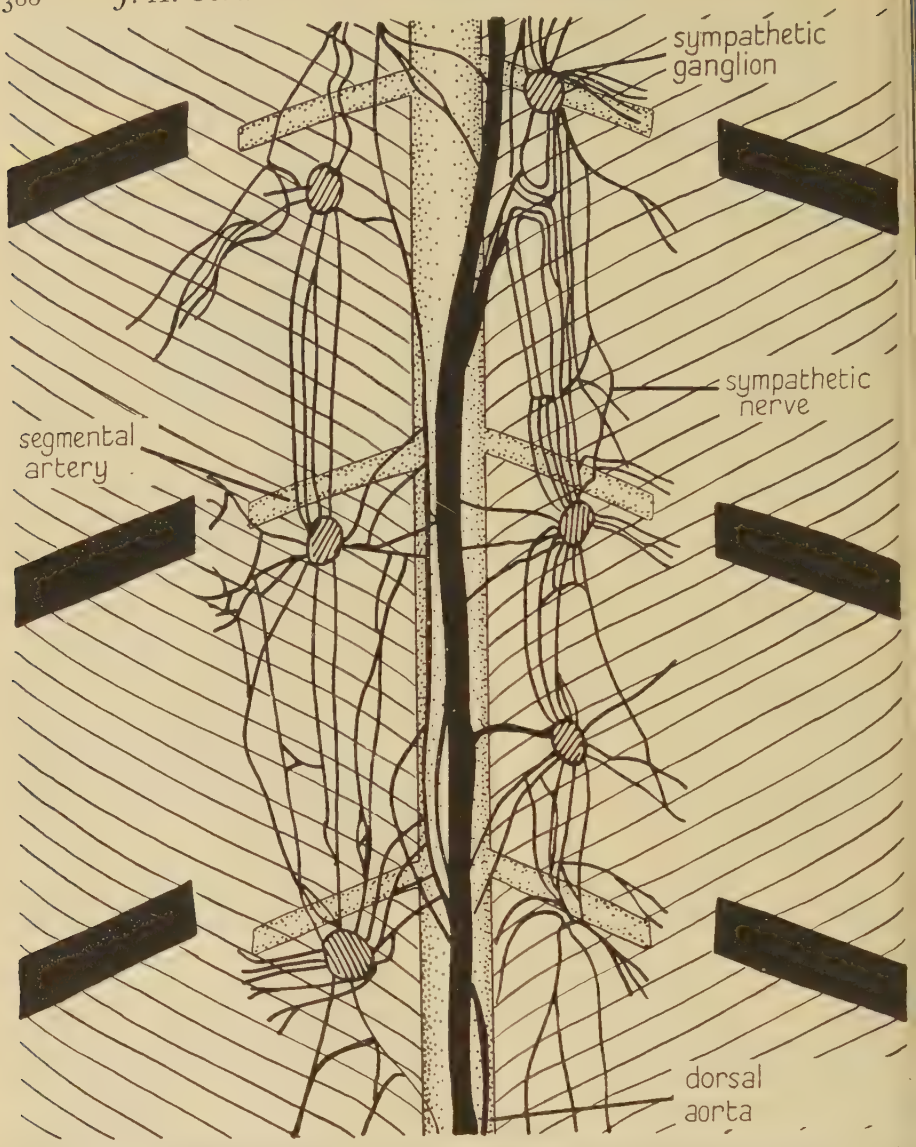
Connectives and Commissures

Not only do the sympathetic ganglia display considerable irregularity in arrangement but there is also a great deal of variation in the sympathetic connectives. There is no definite transverse commissure between ganglia of the same segment. Posterior to the axillary body longitudinally arranged sympathetic strands occur in the dorsal abdominal wall near the median axis (Text-figs. 1, 2, 5, 6). In the anterior abdominal region these nerves are rather scanty, but in the posterior half of the abdomen, above the kidney, they become numerous and conspicuous. The longitudinal strands interconnect with one another to form an irregular plexus which lies lateral and ventral to the dorsal aorta, and which contains the sympathetic ganglia. Longitudinal nerves may link together two successive sympathetic ganglia, or may bypass one or several ganglia. Some nerves do cross the mid-line, but they usually interconnect nerve strands, not ganglia.

The efferent fibres in the sympathetic nerves are small in diameter, and are lightly myelinated or unmyelinated. Occasional sensory fibres occur: these are conspicuous by virtue of their larger diameters and thicker myelin coverings. Many of the fibres in these strands are post-ganglionic axons which travel some distance longitudinally before entering visceral nerves and proceeding to the end-organs.

In dogfishes and rays a similar diffuse and irregular arrangement of sympathetic strands has been noted by several workers. Chevrel (1887) considered that a true sympathetic cord is absent in selachians (*Scyllium* and *Acanthias*). In general all the sympathetic ganglia are united among themselves in a coarse network. However, a connecting cord may be absent between two successive ganglia or, when present, it may be divided into two or three fine strands and have an irregular course. Hoffmann (1900), in a study of the development of *Acanthias*, concluded that longitudinal connectives between ganglia are either absent or are extremely tenuous. Müller (1920) observed longitudinal connectives in the *Squalus* embryo, but noted that they are very thin. In *Raja*, Müller and Liljestrand (1919) found that although ganglia are bound together by longitudinal anastomoses, such connexions vary considerably. Young (1933*a*), in *Scyllium*, &c., noted that ganglia of succeeding segments are sometimes joined together by longitudinal nervous strands, but often no such connexion is present. He concluded that a definite sympathetic chain, such as occurs in teleosts and tetrapods, is certainly lacking.

Fine rami communicantes extend from the spinal nerves to the sympathetic system. Only white rami of lightly myelinated fibres have been observed but, owing to the extreme tenuity of the rami communicantes, the possibility exists that fine grey rami are present and have been overlooked. The rami are segmentally arranged: they arise some distance laterally from the ventral

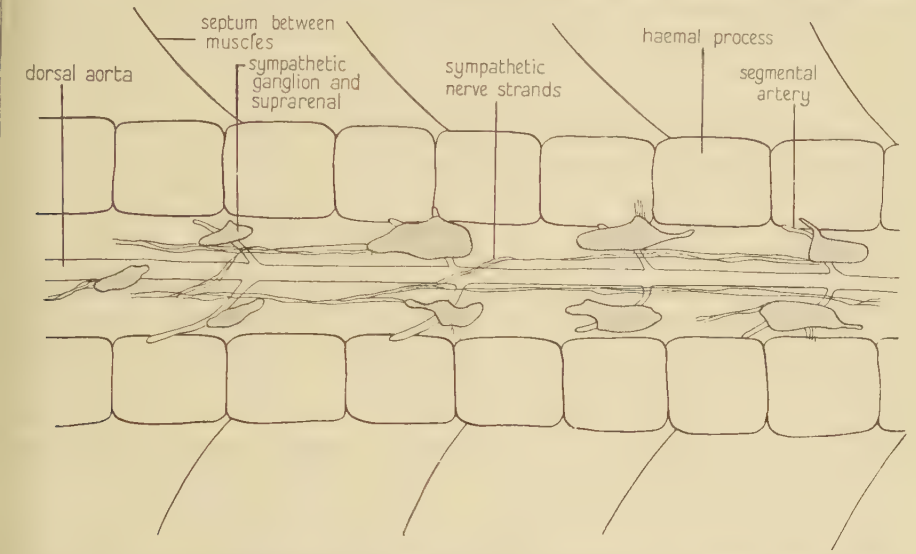


TEXT-FIG. 5. Drawing of dissection. Dorsal body-wall of an adult ♀ ratfish, just in front of the kidney. × 5½.

rami of the spinal nerves, and extend medially to terminate in sympathetic ganglia or the longitudinal sympathetic strands. The large gastric ganglia are composite structures and are connected with a number of anterior spinal nerves. In *Chimaera monstrosa*, Chevrel (1887) found that these ganglia receive at least five rami communicantes from anterior spinal nerves.

The axillary bodies in selachians are similarly of composite origin, as evidenced by their embryonic history and their connexions in the adult. The

gastric ganglia receive a variable number of rami from anterior spinal nerves, the exact number varying with the individual animal and the species (Young, 1933*a*). Chevrel (1887) observed from 10 to 15 rami in *Scyllium*, *Raja*, and *Torpedo*. That the multiplicity of rami results from fusion of a corresponding number of ganglia is shown by the fact that, in embryo *Squalus*, the gastric ganglia are formed by union of sympathetic anlagen of segments 1 to 14 (Müller, 1920). Similarly, in *Acanthias*, sympathetic anlagen of segments



TEXT-FIG. 6. Semi-diagrammatic representation of the sympathetic system as seen in longitudinal sections. Dorsal body wall of a young specimen, in the region of the kidney. Based on superimposed tracings of microscopic projections of serial sections. × 14.

6 to 15 fuse together in varying degrees to form the gastric ganglia during development (Hoffmann, 1900).

The nature of the rami communicantes in selachians has been investigated in some detail by Young (1933*a*). He found that only white rami of pre-ganglionic fibres occur in these forms; such post-ganglionic fibres as do extend to the body-wall accompany the segmental arteries. In some teleosts at least (Young, 1931*a*), and in tetrapods (Gaskell, 1920; Langley, 1921), as is well known, post-ganglionic fibres reach peripheral blood-vessels and dermal structures via recurrent grey rami and the spinal nerves.

Visceral Nerves and Post-ganglionic Pathways

The sympathetic system supplies post-ganglionic fibres to the walls of blood-vessels. These fibres arise from the sympathetic ganglia and proceed to the dorsal aorta or accompany the segmental and visceral arteries. Other nerves leave the sympathetic ganglia and proceed to the abdominal viscera.

The alimentary tract of chimaeroids is peculiar in that there is no stomach, and in that the dorsal mesenteries are reduced to small strands enveloping the

coeliac axis and the two mesenteric arteries. The oesophagus passes directly into the duodenum; the wall of the former contains only striated muscle. Following the short duodenum there is a long ileum containing a spiral valve. Behind the ileum there is a short rectum which ends at the anus. The walls of the rectum contain smooth muscle. The urogenital apertures are separate and there is no cloaca.

The coeliac axis supplies the posterior oesophagus, duodenum, liver, and pancreas, and sends branches to the anterior ileum. The anterior mesenteric artery crosses the right face of the pancreas, to which it sends branches, gives rise to the splenic artery, and provides arteries for the dorsal and ventral surfaces of the ileum. The posterior mesenteric artery runs to the dorsal surface of the posterior ileum. Accompanying these several visceral arteries are cords or bands of smooth muscle, particularly between the pancreas and ileum where the muscle forms two flat bands of considerable magnitude. The muscular bands act as slings, suspending the ileum and neighbouring viscera within the abdominal cavity.

Post-ganglionic neurones and a myenteric plexus occur just within the serosa in the duodenum and ileum, but not in the oesophagus or rectum. A submucosal plexus is also located in the ileum. The vagal supply to the oesophagus may be classified as a special visceral efferent. The innervation of the rectum presents certain peculiarities which are discussed below.

The gastric ganglia give rise to the anterior splanchnic nerves in the following manner. Two or three main nerves and several smaller twigs originate from the median face of each gastric ganglion, and these nerves extend to the coeliac axis, where they form a plexus or network in the wall of the artery. Although the right and left splanchnic nerves combine in a complex manner, there is no direct connexion or commissure between the bilateral gastric ganglia. Occasional nerve-cells occur along the course of the splanchnic nerves near their origin. The splanchnic nerves resolve themselves further distally into two main trunks lying in the wall of the artery. After giving off fibres to the ductus choledochus and anterior pancreas, the splanchnic nerves accompany the coeliac artery to the duodenum and ileum (Text-fig. 2; Pl. I, fig. 6).

The intestine, liver, pancreas, and spleen appear to receive their sympathetic innervation nearly or entirely from the anterior splanchnic nerves which accompany the coeliac artery. No comparable splanchnic nerves arise from more posterior sympathetic ganglia to accompany the anterior and posterior mesenteric arteries. On the other hand there are occasional groups of nerve-cells and stout bundles of nerve-fibres along the lower course of the anterior mesenteric branches, towards the ileum. These nerves probably proceed from the wall of the ileum to the bands of smooth muscle about the anterior mesenteric artery. There are no distinct visceral nerves along the course of the posterior mesenteric artery.

A comparable arrangement of splanchnic nerves occurs in selachians. In *Scyllium* and *Raja* there are two gastric ganglia which are independent of

each other. Each gives rise to about three anterior splanchnic nerves which bear scattered nerve-cells on their proximal course and which continue to form a plexus about the coeliac artery. They run with the coeliac artery to supply the oesophagus, stomach, pylorus, duodenum, anterior ileum, liver, bile passages, and spleen (Babkin *et al.*, 1935; Chevrel, 1887; Müller and Liljestrand, 1919; Young, 1933*a*). But in addition there are middle splanchnic nerves which arise from the sympathetic ganglia of several segments, and which accompany the anterior mesenteric artery to the ileum, spleen, and possibly the pancreas (op. cit.). Posterior splanchnic nerves run as separate strands in the mesentery and along the posterior mesenteric artery to the colon and rectum (Müller and Liljestrand; Young).

The splanchnic nerves of *Hydrolagus* thus reveal some distinctive features in organization and distribution, when compared with those of selachians. Apart from the absence of a stomach and of corresponding gastric nerves, the most noteworthy feature is the absence of middle and posterior splanchnic nerves at the levels of the mesenteric arteries. Probably the explanation of these differences is to be sought in the loss of the stomach, reduction of dorsal mesenteries, and shortening of the gut and abdominal cavity (Barrington, 1942; Dean, 1906). It is noteworthy, in both *Hydrolagus* and selachians, that there are no coeliac and mesenteric ganglia on the course of the splanchnic nerves, which contain only post-ganglionic fibres.

The sympathetic supply to the kidney is confined to nerve-fibres which pass from the sympathetic ganglia along the renal arteries. No nervous termination has been demonstrated on renal tubules or glomeruli. There is some histological evidence for innervation of renal units in mammals (Maximow and Bloom, 1944), but not for selachians (Young, 1933*a*). The sympathetic also innervates the Müllerian ducts and vasa deferentia. The visceral nerves to these structures arise medially from the segmental ganglia; they proceed between the kidney and the subcardinal veins across the ventral face of the kidney, and terminate in a plexus in the walls of the ducts. Similarly, in *Scyllium*, Young (1933*a*) found that the sympathetic ganglia send nerves to the oviducts and vasa deferentia in each segment.

There are no sympathetic fibres to the heart.

At the posterior end of the abdominal cavity several small twigs arise from the ventral rami of three or four spinal nerves. These twigs contain small myelinated fibres which enter the wall of the posterior rectum near the anus, at the level of the pelvic cartilage. Sections show that these nerve-fibres proceed directly to smooth muscle in the rectal wall. In *Scyllium* and *Torpedo* the walls of the cloaca are innervated directly from several spinal nerves in the posterior abdomen (Young, 1933*a*). This innervation of the posterior extremity of the abdominal canal is essentially the same in both chimaeroids and selachians. In neither group are there post-ganglionic cells in the nervous pathway; this feature excludes it from the autonomic system *sensu stricto*. Young has suggested the possibility that it may correspond to the anal sphincter nerves of *Uranoscopus* (Teleostei), and perhaps to the pudendal

nerves of tetrapods, but an homology is difficult owing to the absence of autonomic ganglia and peripheral neurones.

Relation between Sympathetic and Suprarenal Tissues

It is well known that in lower vertebrates the cortical and medullary portions of the adrenal glands are separate from one another and the medullary tissue is closely associated with the sympathetic system (Kendall, 1947). In *Hydrolagus* the cortical or interrenal tissue forms a long median block lying beneath the dorsal aorta and between the two mesonephroi in the posterior half of the kidney. Besides the main interrenal mass there are several smaller aggregations of interrenal tissue lying more anteriorly (Text-fig. 3; Pl. I, figs. 2, 3). The interrenal tissue receives no sympathetic supply. The suprarenal tissue occurs as segmentally arranged bodies associated with the sympathetic ganglia from the axillary body to the level of the anus (Pl. I, figs. 1, 2, 3, 5; Pl. II, fig. 7).

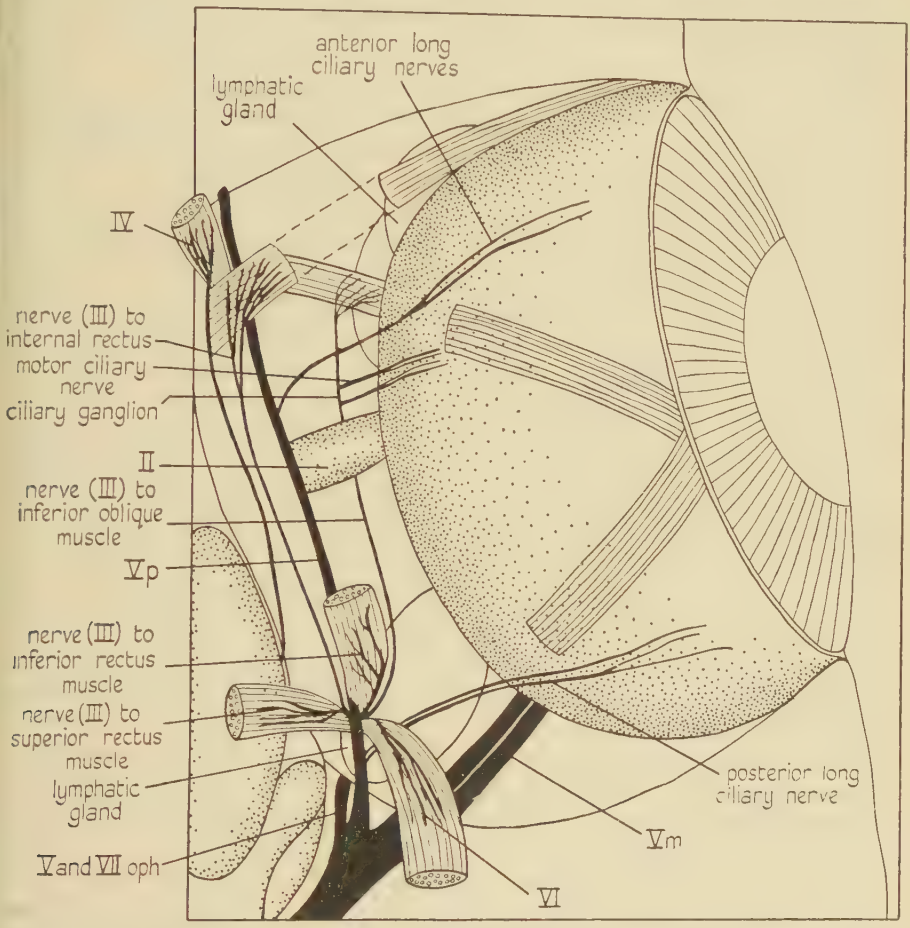
The axillary bodies contain the largest mass of suprarenal tissue in the body. They completely envelop the subclavian arteries and are suspended in the posterior cardinal sinuses. Each body is made up of densely packed suprarenal cells which are irregular or stellate in shape and which bear long processes. The cytoplasm is basiphilic and the nuclei are rather small and densely staining. Nerve-fibres entering this body proceed among the suprarenal cells. The smaller gastric ganglion is attached to the medio-ventral face of each suprarenal mass. Occasional sympathetic cells are also distributed about the periphery of the suprarenal body, and within the latter structure (Text-fig. 2; Pl. I, figs. 1, 5; Pl. II, fig. 7).

Small aggregations of suprarenal tissue are associated with the anterior sympathetic ganglia. Within the kidney region the suprarenal bodies and the sympathetic ganglia are inextricably fused, forming conjoined ganglio-medullary units. The suprarenal (chromaffin) cells are rather small with fairly regular cell boundaries. They have basiphilic cytoplasm, and small densely staining nuclei. Besides post-ganglionic fibres to the blood-vessels and viscera, the ganglionic cells send numerous nerve-fibres into the suprarenals where they terminate on or among the medullary cells.

Cranial Autonomic System

Midbrain Outflow. The first autonomic (parasympathetic) pathway in *Hydrolagus* is represented by pre-ganglionic fibres which run in the oculomotor (IIIrd cranial) nerve to the ciliary ganglion. Since a cranial sympathetic system is absent, the ciliary ganglion receives no sympathetic contribution. The oculomotor is a motor nerve, containing both somatic efferent and general visceral efferent fibres. General visceral afferent fibres destined for the same peripheral region are contained in the ramus ophthalmicus profundus. The latter is relatively large in this fish and extends anteriorly across the lateral wall of the orbit below the oculomotor branch to the anterior rectus muscle. It constitutes a dorsal root corresponding to the same cranial segment as the oculomotor.

The ciliary ganglion lies on the ventral branch of the oculomotor nerve proceeding to the inferior oblique muscle, and it is so closely applied to this nerve that no separate radix brevis is distinguishable (Text-fig. 7; Pl. II, figs. 10, 11). The ganglion itself is situated distally towards the insertion of



TEXT-FIG. 7. Drawing of dissection of the orbit. Adult ratfish; dorsal view. Legend: II, optic nerve; IV, trochlear nerve; V_m, maxillary and mandibular branch of the trigeminus; V, VII_{oph}, superficial ophthalmic branch of trigeminal and facial nerves; V_p, ramus ophthalmicus profundus; VI, abducens. ×2·2 (*ca.*).

the nerve into the inferior oblique muscle, but there are also scattered nerve-cells elsewhere on the course of the nerve. Fine non-myelinated nerves arise in the vicinity of the ganglion and pass to the wall of the eyeball: these are motor ciliary nerves (short ciliary nerves). There are no ganglion cells on the root or other branches of the oculomotor nerve.

The profundus nerve crosses the root of the oculomotor where the latter pierces the lateral wall of the orbit, and it lies on top of the oculomotor root

and the origin of the common ventral ramus to the inferior rectus and inferior oblique. There is a large sensory ganglion on the profundus at this level. The profundus nerve and ganglion and the ventral ramus of the oculomotor nerve are actually fused together at this point by common connective tissue, and it is impossible to separate them cleanly in a dissection. However, the profundus sends no nerve-fibres to the oculomotor, and a radix longa is absent. This point has been checked carefully both by dissections and by serial sections of the entire orbital region.

The profundus gives off two sets of medullated sensory nerves to the eyeball. These are the posterior and anterior long ciliary nerves (Text-fig. 7; Pl. II, fig. 9). The posterior arise as a set of small nerves from the region of the profundus ganglion, and extend over the posterior surface of the eyeball. The anterior arise from the profundus well forwards towards the superior oblique muscle and pass to the wall of the eyeball. These nerves pierce the sclera through small apertures in the cartilage investing the eyeball. In *Hydrolagus*, therefore, the visceral sensory and motor fibres follow an independent course to the eyeball. Young (1933*a*) has described similar anterior and posterior long ciliary nerves in *Mustelus*.

In *Chimaera monstrosa* there is a ganglion or small group of nerve-cells on the ventral ramus of the oculomotor and a ciliary nerve is given off to the eyeball from this point (Cole, 1896; Schwalbe, 1879). But in addition to this ganglion Cole has figured a discrete ciliary ganglion, which lies near the proximal course of the ventral ramus, and which is connected both with this ramus and with the profundus by a distinct radix brevis and r. longa, respectively. Ciliary nerves proceed to the eyeball from the ciliary ganglion and the profundus nerve. The first (Schwalbe's) ganglion corresponds to the ciliary ganglion described in *Hydrolagus*; but a second distinct ciliary ganglion, connected with oculomotor and profundus nerves by radices, as described by Cole, is absent in *Hydrolagus*.

The ciliary complex has been studied in some detail in other elasmobranchs. In *Mustelus* and *Squalus* a ciliary ganglion lies on the oculomotor just within the orbit. Additional nerve-cells and groups of cells occur on the ventral ramus of the oculomotor and on the short ciliary nerves. The latter take their origin from the oculomotor nerve and ciliary ganglion, and form a ciliary plexus which proceeds to the eyeball. The profundus sends a branch to the ciliary plexus, and separate sensory nerves to the eye (Norris and Hughes, 1920; Young, 1933*a*). In *Scyllium*, on the other hand, sensory and motor roots from the profundus and oculomotor join to form two mixed ciliary nerves which supply the eyeball, in part. The ciliary ganglion is represented by three groups of cells on ventral branches of the oculomotor and on the short ciliary nerves which form a ciliary plexus (Schwalbe, 1879; Young, 1933*a*).

A ganglion has been found on the trochlear nerve in some small specimens of *Hydrolagus*. When present it lies closely joined to the nerve shortly after the latter pierces the dorso-lateral cranial wall. This ganglion possibly repre-

sents an ephemeral feature of ontogeny, since it is absent in the adult, and may not even be present bilaterally in the same immature specimen. No nerve-cells are present on the course of the abducens nerve.

A transient trochlear ganglion has been reported in *Squalus*. Neal (1914) found a group of cells at the point of union of anlagen of the trochlear and superficial ophthalmic nerves: this group of cells appeared to be a rudimentary autonomic ganglion comparable to the ciliary ganglion which develops on the oculomotor. The trochlear ganglion disappears in the adult. No ganglion develops on the abducens in this form.

Hindbrain Outflow. Ganglionic cells occur in post-trematic rami of the glossopharyngeal and vagal nerves. These cells lie scattered among the nerve-fibres, or are aggregated into small ganglia along the course of the nerves. The cells are encapsulated and are associated with bundles of fine nerve-fibres (Pl. II, fig. 12). They constitute post-ganglionic autonomic neurones concerned with innervating smooth muscle in the branchial and pharyngeal regions. A careful search of serial sections was made for autonomic ganglia on other cranial nerves. Ganglionic autonomic cells are not present in pre-trematic branches of the glossopharyngeal and vagal nerves, in the hyomandibular or hyoidean branches of the facial, in the mandibular branch of the facial, or in the trigeminal nerve.

Autonomic ganglia have been identified on post-trematic rami of the branchial nerves in *Squalus*, *Mustelus*, and *Scyllium*, but not on the trigeminus (V). Scattered cells and small ganglia occur on the hyomandibular branch of the facial, on the ramus hyoideus, and on the post-trematic branches of the glossopharyngeal and first three branchial rami of the vagus. Autonomic ganglia are absent from the fourth branchial vagal nerve. From the ganglia arise bundles of small non-medullated fibres which have a somewhat diffuse arrangement (Allis, 1901; Norris and Hughes, 1920; Young, 1933*a*).

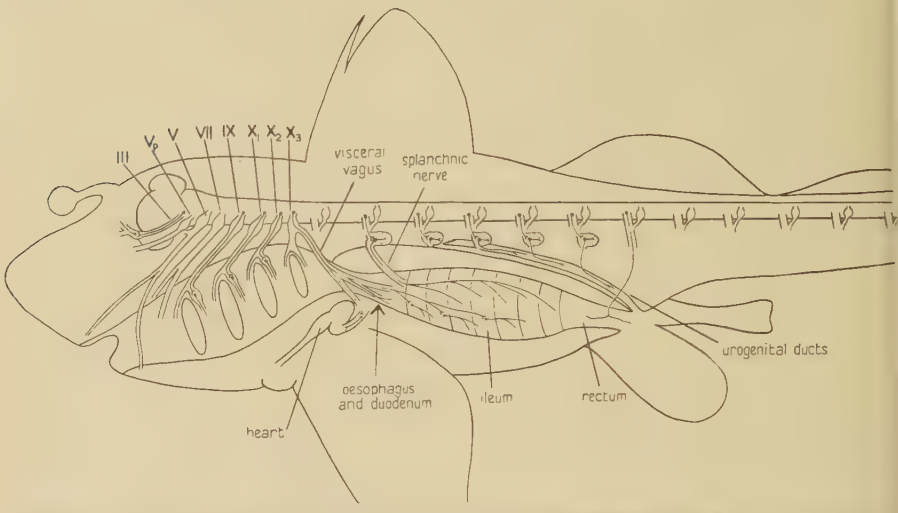
In *Hydrolagus* the visceral branches of the right and left vagi supply the anterior alimentary canal and the heart. From each visceral vagal nerve a cardiac branch descends ventrally in the wall of the duct of Cuvier to the sinus venosus. The cardiac nerves give rise to a network of fibres in the walls of the ducts of Cuvier, sinus venosus and at the sino-atrial junction, but both nerve-cells and fibres are lacking in the ventricle (Pl. II, fig. 8).

The visceral vagal nerves extend from the dorso-lateral body-wall into the wall of the oesophagus, where they immediately subdivide into numerous branches passing anteriorly and posteriorly. Some anterior branches pass dorsally to reach the pregastric ganglia, or extend forwards into the pharynx. Posterior branches form discrete bundles which course longitudinally in the pigmented layer of the serosa; at intervals they give off fascicles which descend into the muscular layers of the oesophagus, where individual nerve-fibres terminate on striated muscle-fibres by motor-end plates. The vagal fibres continue posteriorly in the gut, and are distributed to the wall of the intestine. A vagal contribution to the sympathetic system has been described above (p. 385).

The course of the visceral vagus in the ratfish corresponds closely with that found in selachians. In *Scyllium* and *Raja* the vagus innervates the oesophagus, the corpus, and the pyloric region of the stomach (Babkin *et al.*, 1935; Young, 1933*a*). According to Müller and Liljestrand (1919), the vagus reaches the ileum in *Raja*, and Müller (1920) traced it to the intestine in developing specimens of *Squalus*.

Discussion

All the facts assembled in this investigation demonstrate an essential similarity between the autonomic nervous systems of chimaeroids and sela-



Text-fig. 8. Diagram of the autonomic nervous system of *Hydrolagus colliei*. Roman numerals, cranial nerves. V_p, ramus ophthalmicus profundus.

chians (Text-fig. 8). In both groups the sympathetic ganglia are segmentally arranged in the abdominal region, and are connected with the spinal nerves by white rami communicantes, but segmental correspondence is not strict in the adult, and there may be more than two ganglia to each segment. Of greater interest is the absence of definite sympathetic trunks and commissures, with the result that the sympathetic system has a rather diffuse organization. Axillary bodies and gastric ganglia are peculiar to these two groups, and the anterior splanchnic nerves are similarly arranged. A system of sympathetic ganglia and connectives is absent from the tail of both groups, although transient ganglia do appear ontogenetically, in the caudal region of selachians at least. Other primitive features are the absence of a cephalic sympathetic component, of sympathetic cardiac nerves, and of collateral sympathetic ganglia. A sacral outflow (parasympathetic) is also wanting.

The close correspondence of autonomic organization in these fish points to a basic pattern derived from a common ancestral form. The Holocephali are an ancient group which have had an evolutionary course separate from

the Selachii since the Devonian, and probably are derived from early selachian stock through the palaeozoic bradyodonts. Modern forms, the Euselachii and the Chimaeridae, occur from the Jurassic (Woodward, 1932; Moy-Thomas, 1939; Berg, 1947). Extant chimaeroids show many peculiar anatomical features, such as holostylic jaw suspension, operculum, cephalic clasper in the male, lack of a spiracle, peculiar dentition, absence of a stomach, &c. All these characters are probably secondary, and not primitive in nature (Dean, 1906), although Fahrenholz (1915) regarded the absence of a stomach as a primitive character. The autonomic system, in contrast, appears to have undergone remarkably little alteration, except with regard to secondary changes such as disappearance of a stomach and reduction of mesenteries in chimaeroids. Its basic character in these two groups, chimaeroids and selachians, seems well attested by these dual lines of evidence, and it constitutes a simple and primitive level of organization.

The organization of the autonomic system in elasmobranchs (Selachii and Holocephali) stands in an interesting position to that found in teleostomes and tetrapods. Chevrel (1894) has described this system in the sturgeon (*Acipenser*). In this fish there is an irregular sympathetic plexus in the abdomen. The plexus contains segmentally arranged ganglia, and it is connected at regular intervals to the spinal nerves by rami communicantes. Sympathetic ganglia are absent from the head and tail. From this account it appears that the sympathetic system is no more highly organized in *Acipenser* than it is in elasmobranchs.

In teleosts the sympathetic ganglia are segmentally arranged in a definite chain with connectives and commissures. Both white and grey rami communicantes are present. Sympathetic ganglia are present in the tail, and the sympathetic system extends into the head to connect with the parasympathetic system (Chevrel, 1887; Young, 1931*a*). Thus in teleosts, as in Amphibia, the autonomic system shows considerable advances in complexity and regularity of organization over the same system in elasmobranchs. Moreover, it is probable that the relatively diffuse systems of elasmobranchs and Chondrostei represent a simple level of organization from which the more highly organized systems of higher teleostomes and Amphibia evolved.

Gastric physiology of lower vertebrates has been reviewed by Barrington (1942), and further accounts of autonomic functioning in fish have been given by Lutz (1930), Young (1931*b*, 1933*b*, 1933*c*), and Babkin (1946). No information is available for chimaeroids, but in selachians it has been shown that both the sympathetic and parasympathetic systems are motor to the gut, and that there is no functional antagonism between these two components. In teleosts there is an increase in the field of autonomic innervation (air bladder, chromatophores), and of double innervation (cephalic structures), and evidence for some functional antagonism (eye) (Bohr, 1894; Young, op. cit.; Waring, 1942, review). Autonomic functioning is on a relatively simple level in elasmobranchs and parallels simplicity of structure. With increase in morphological complexity in teleosts and tetrapods there is,

pari passu, development of greater physiological diversity in the autonomic system.

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EXPLANATION OF PLATES

Plate I

Fig. 1. Transverse section of the body at the level of the oesophagus. The paired axillary bodies are shown adjoining the subclavian arteries. Young ratfish. Holmes's silver.

Fig. 2. Transverse section of the body in the posterior kidney region. Young ratfish. Holmes's silver.

Fig. 3. Transverse section through the dorsal body-wall in the posterior abdominal region. Young ratfish. Haematoxylin and eosin.

Fig. 4. Transverse section through the dorsal body-wall in the middle abdominal region. Young ratfish. Haematoxylin and eosin.

Fig. 5. Longitudinal section through axillary body (transverse to body axis). Young ratfish. Holmes's silver.

Fig. 6. Transverse section of the dorsal body-wall in the anterior abdomen. Young ratfish. Holmes's silver.

Plate II

Fig. 7. Transverse section through axillary body of an adult ratfish. Holmes's silver.

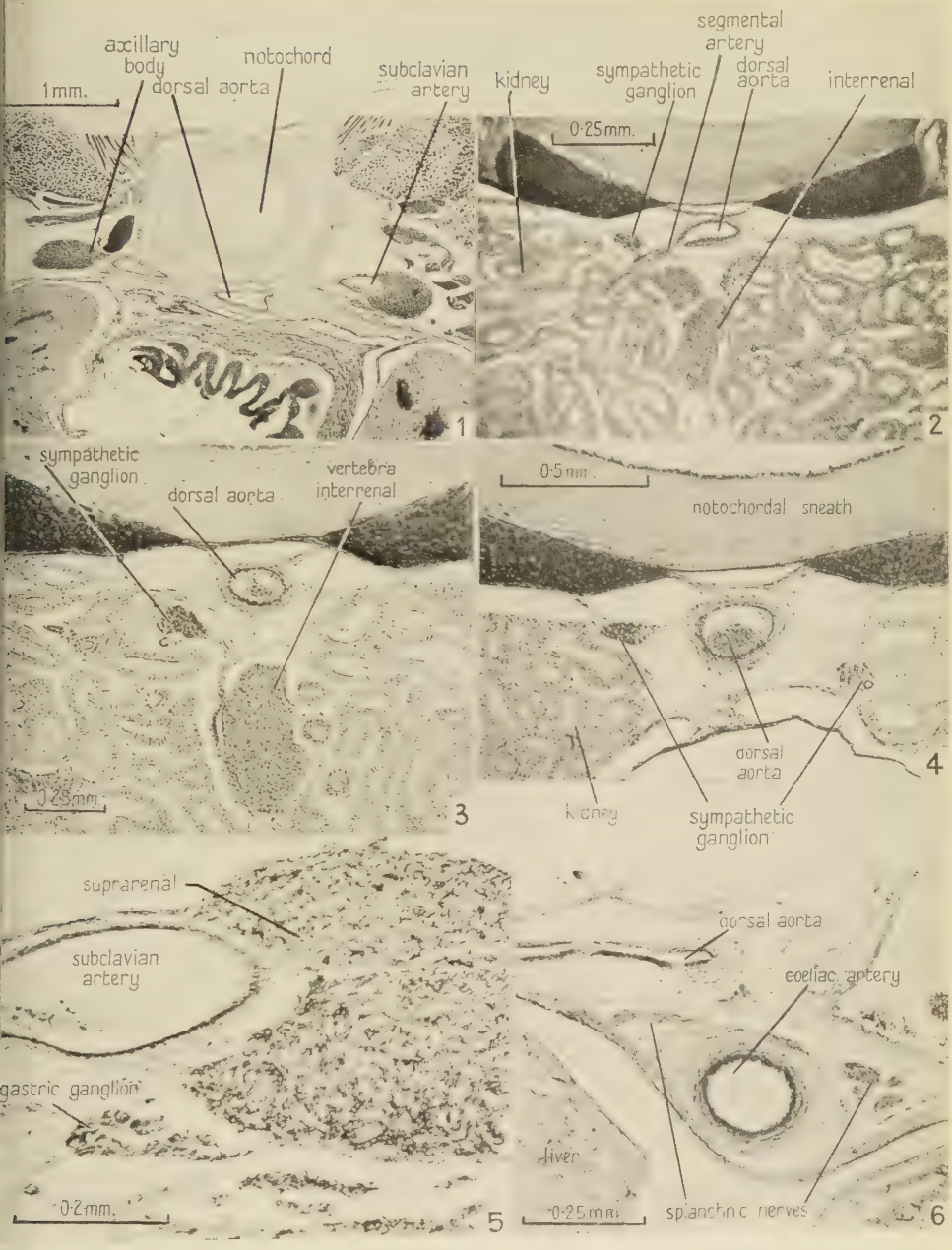
Fig. 8. Transverse section through the wall of the sinus venosus to show a branch of the cardiac vagal nerve, and cardiac nerve-cells. Young ratfish. Holmes's silver.

Fig. 9. Transverse section through the orbit of a young ratfish. Holmes's silver. Legend: gang p, profundus ganglion; III, ventral ramus of the oculomotor nerve; V_p, ramus ophthalmicus profundus.

Fig. 10. Longitudinal section through the oculomotor branch to the inferior oblique muscle (III). Adult ratfish. Holmes's silver.

Fig. 11. Transverse section through the orbit of a young ratfish. Holmes's silver.

Fig. 12. Section cut longitudinally along the post-trematic branch of the second branchial vagal nerve (X^2). Adult ratfish. Holmes's silver.



J. A. C. NICOL—PLATE I

J. A. C. NICOL—PLATE II

Determination of the Form of Regenerating Limbs in *Asellus aquaticus*

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Summary

1. The nerve-supply to the regenerating anterior pleopods of *Asellus aquaticus* influences the rate of regeneration but not the character of the regenerate.

2. The tissues of the locality of the blastema determine the character of the regenerate and also influence the rate and extent of regeneration.

3. In contrast to the thoracic limbs these appendages are very sensitive to the induction of abnormalities of regeneration.

4. The complete suppression of regeneration of a limb is the most common induced abnormality. An all-or-none effect is evident.

5. The development of supernumerary structures is the next most common abnormality.

6. The re-emergence of the endopodite of the first pleopod, suppressed in normal ontogeny, is the most frequent supernumerary. It often shows homoeotic differentiation.

7. A tendency towards bilateral symmetry is evident in all abnormalities, in spite of asymmetry in experimental treatment.

8. Fusion of individual blastemae is not uncommon. There is no fusion with homoplastic embryonic tissue.

9. There is no substance with the properties of a 'wound hormone'.

10. The regeneration of Pl. 2 in the male is more easily suppressed than that of Pl. 1.

Introduction

IT has been demonstrated (Needham, 1945, 1946) that innervation of the blastema is essential for the normal rate of regeneration of thoracic limbs in this Isopod Crustacean. The classical experiments of Herbst (1896, 1899) implied that the nerve-supply to a Crustacean appendage might determine also the nature of the regenerate: that the presence or absence of the optic ganglion might determine respectively the normal regeneration of the eye or its replacement by an antenna. Atypical regenerates are not infrequently produced experimentally (Korschelt, 1927; Wolsky, 1932; Needham, 1941*b*, &c.) and such homoeotic appendages (i.e. appendages characteristic of some other segment of the body) are sometimes observed in the field (Bateson, 1894; Needham, 1942*b*, &c.). Nevertheless, the regenerating limb of a Crustacean normally recapitulates its typical form with remarkable fidelity. The general properties of the nervous system, and its high degree of morphological differentiation, are consistent with the possibility that the nature of an appendage might be determined in regeneration by its nerve-supply. The

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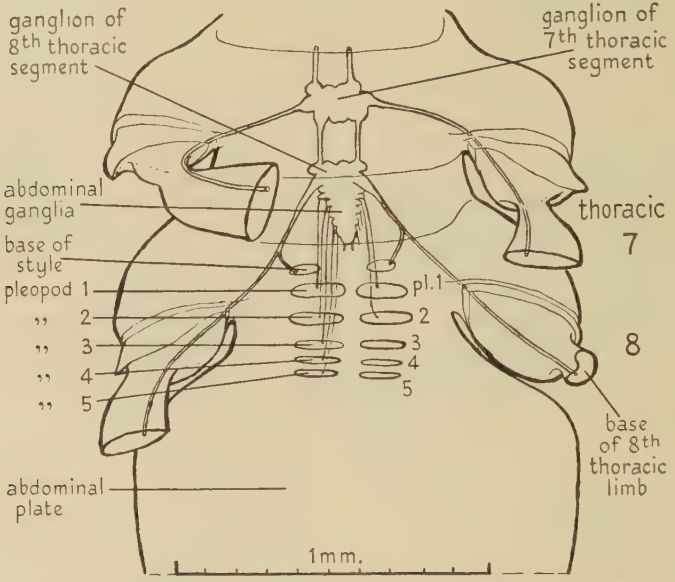
evidence from animals in general (Korschelt, 1927, pp. 600–2) is scanty and conflicting. The vascular system, the only other general system of the body which might influence the quality of the regenerate, almost certainly lacks the necessary static basis for the determination of regionally specific limb-form, and it seems clear that the determination must be due either to the nervous system or to properties of the tissues locally at the side of the regeneration-blastema. It has been shown that the rate of regeneration is dependent on



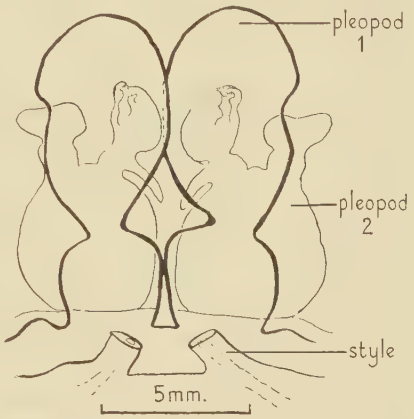
TEXT-FIG. 1. Outline drawing of posterior thoracic and anterior abdominal portions of ventral surface of male *Asellus aquaticus*, to show positions of attachment of limbs to body-wall, and the innervation of the limbs seen by transparency.

factors in these local tissues (Needham, 1947, 1949*b*), as well as upon innervation. In the present experiments it was hoped to elucidate the respective roles of these two possible determinants of the nature (i.e. quality) of a regenerate.

METHODS

If the nature of a regenerate is in fact determined by the nerve-supply to that limb, then it should be possible to induce an atypical regenerate by diverting to a regeneration-blastema the peripheral nerve of another limb. The thoracic limbs of this animal, used in previous studies (Needham, 1945, 1946, 1947, 1949), are unsuitable for this purpose, not only on account of the difficulty of diverting the nerve-supply from a neighbouring segment, but also because neighbouring limbs differ in little but relative proportions (Needham, 1943), which change continuously during regeneration and show great individual variations. By contrast the first two pairs of pleopods (subsequently designated Pl. 1 and Pl. 2) in the male *Asellus aquaticus* L. are ideal (cf. Need-

ham, 1941*b*) for the present experiments. Each has a characteristic, highly differentiated form (see, e.g., Needham, 1938) shown in outline in Text-fig. 2, and the four appendages are situated in close proximity so that their nerves are correspondingly approximated (Text-fig. 1) and are therefore potentially ideal material for the induction of errant re-innervation, following the simultaneous transection of two or more of them. The ganglia of the abdominal segments are concentrated in the eighth thoracic segment (Text-fig. 1) and the peripheral nerves to Pl. 1 and 2 are therefore relatively long, thus facilitating their transection, and increasing the expectation of their subsequent regeneration along an errant course.



TEXT-FIG. 2. Outline drawings of the first two pairs of abdominal appendages of *Asellus aquaticus*, male. Setae omitted and styles cut away for clarity. First pair in heavy outline.

The individual nerves are not always clearly visible, even under critical illumination, in the living animal and transection demands accurate previous knowledge of their course. This, and all other operations, were performed under a high-power dissection microscope. A fine recurved steel needle, sharpened to give both a point and a cutting edge, was used for nerve transection, ensuring minimal damage to the exo-skeleton and minimizing haemorrhage and infection. The success of the operation was gauged by the paralysis of the limbs and confirmed by histological examination of a typical sample. Both uni- and bilateral transection of the two nerves were practised.

In other experiments the ganglia supplying these nerves were destroyed as a check on the possibility that nerve-transection might also result in permanent denervation of some of the limbs. The ganglia are sufficiently bulky (Text-fig. 1) and opaque to be visible in the living animal. The isolation of the ganglia of the posterior segments of the abdomen from the rest of the nervous system by bilateral ganglionectomy does not completely inactivate the gills (pleopods 3 to 5) and, moreover, in shallow water the animal respires adequately through the general exo-skeleton, even with the gills inactive. Mortality was somewhat higher in these experiments than in those not involving denervation (Tables 1, 4) but not markedly so (p. 413). Transection of the peripheral nerves sometimes involved also the nerves to the anterior gills.

Following operation to the nerve-supply the appendages Pl. 1 and 2 were amputated cleanly with fine sterilized scissors, leaving one-sixth of the appendage as stump (p. 411).

As control experiment simple amputation of the four limbs was considered adequate. The more precise control, involving incision of the body-wall without

nerve transection, was avoided because of the danger of accidental damage to the nervous system. As part of the control experiments for this and for the following series, the effect of varying the level of amputation, along the appendages, was examined.

The alternative possibility that the form of the regenerate depends on properties of the tissues at the site of the blastema (p. 402) was examined in a number of ways, following amputation of the appendages: (*a*) an attempt was made to subdivide the regeneration blastema by deep longitudinal incisions into the stumps of the appendages; (*b*) the tissues of the stump were extensively lacerated in order to increase the amount of damaged tissue (? and of 'wound hormone') and also to induce regeneration at an irregular surface (cf. Przibram, 1921); (*c*) the body-wall between the bases of the four limbs was ablated, with the object, rendered possible by the proximity of the four appendages, of inducing fusion between blastemae; (*d*) homoplastic tissue, chiefly from embryos, was implanted below the body-wall, between the bases of the appendages, as a possible mechanical or physiological obstacle to the normal organization of regeneration; (*e*) chemical agents were applied to the surface of amputation; (*f*) the varied level of amputation in the 'control' series of experiments may be regarded as a further type of experiment in this group, designed to detect local variation in regeneration potency (Needham, 1947). It should be realized that the variety of these experiments is not indiscriminate but represents the logical development of the series of experiments, which would have been evident in serial publication. The pooling of results is justified (p. 409) on grounds other than those of brevity. This whole group of experiments may conveniently be designated the 'local tissue' (pp. 409–12) series and the other experimental group the 'innervation series' (pp. 412–14). The control experiments for both groups have been pooled (Table 1) as the 'control series' (pp. 405–8).

The female is less suitable for these experiments since (*a*) the first pair of pleopods are absent (Needham, 1938), and (*b*) the second pair are very simple in form (cf. p. 403), and (*c*) the latter are widely separated from each other at their bases (Text-fig. 3). On the other hand, a more striking contrast to the arrangement in the male could scarcely be found and experiments were therefore performed on a limited number of females, as controls for (*a*) the complexity of, and (*b*) the proximity of the male appendages. Moreover, the wide separation from the mid-ventral line of the bases of the Pl. 2 pair, and the absence of the Pl. 1 pair facilitate the transaction of a single peripheral nerve as a control for the more complex operation in the male (p. 403).

The general technique, anaesthetic, &c., were as in previous work (Needham, 1945). In all 807 males and 212 females were used, so that the results may be treated statistically. Operations were performed on batches of 10–20 animals, at various seasons, over a number of years, and the experimental 'errors' may therefore be regarded as approaching a normal distribution.

Regeneration of these appendages was virtually finished in two complete stadia following amputation, under optimum conditions, so that the maximum

rate of regeneration is not greatly different from that of thoracic limbs, though differentiation is considerably less advanced at eclosion (i.e. when the limb is first released) in Pls. 1 and 2 than in the latter. An appendage which was not visible externally after the *second* post-amputational ecdysis was never observed to regenerate subsequently, and was recorded as a complete failure. Observation of small and excessively retarded regenerates was continued until they ceased to differentiate further.

Results

Control Experiments, Simple Amputation

Perhaps the most interesting and unexpected results of these experiments was the very high percentage of both quantitatively and qualitatively atypical regenerates (Table 1), and it is therefore essential to analyse the results of this series first, as a background for the analyses of those of the two experimental series. The high incidence of atypical regeneration stands in sharp contrast to its rarity in regenerating thoracic limbs. Among the 670 normal regenerates of the seventh thoracic limb (Needham, 1949), and the large number of experimentally treated regenerates of this limb (Needham, 1945–9, and others unpublished), there were fewer than ten atypical limbs and complete failure to regenerate was observed only in individuals from which the limb-base was completely extirpated. The atypical regenerate lacked one or more segments, but always remained a typical thoracic limb. Significant for the explanation (p. 417) of this difference between thoracic and anterior abdominal limbs is the fact that the Pls. 1 and 2 appendages of the male lack a preformed autotomy plane [cf. the fifth thoracic limb of the male (Needham, 1942 *b*), which is also used in coition] and probably rarely regenerate in the field.

The most important atypical conditions observed among 'control' regenerates of Pls. 1 and 2 are analysed in Table 1. They may be classified as follows:

1. Deficiencies: the complete failure of regeneration of whole limbs, or of parts of limbs.
2. Excesses: the development of supernumerary limbs, or of parts of limbs.
3. Homoeotic heteromorphs: structures recognized as typical of some other segment of the body.
4. Fusions: between appendages, either between the two members of a pair or between Pls. 1 and 2 of the same side of the body.

Deficiency was by far the most common condition. Whole limbs failed more frequently than parts of limbs and the whole set of limbs failed more frequently than 3, 2, or 1 limbs of the set. There was thus an all-or-none tendency, both within and among the limbs. The incidence of the deficiency conditions increased with the number of limbs involved. Further, symmetrical deficiency conditions were more frequent (40 : 22) than asymmetrical conditions,

although there are only three possible symmetrical conditions, viz. (1) both Pl. 1, (2) both Pl. 2, and (3) all four limbs, absent, as compared with 12 possible asymmetrical conditions, viz. four possible combinations each for the

TABLE 1. *Analysis of Results of Experiments on Regeneration of first two pairs of Abdominal Appendages (Pl. 1 and 2) in the male* Asellus aquaticus *(L.)*

(1) Following simple amputation (Control Series).
(2) Following varied treatment of tissues locally, after amputation ('Local-tissue' Series).
(3) Following ganglionectomy or nerve transection at the time of amputation ('Innervation' Series).

Nature of the effect	*Control series*	*Local-tissue series*	*Innervation series*
I. *Deficiency of:*			
1. All four limbs	17	58	6
2. Both Pl. 2 and one Pl. 1	10	17	4
3. Both Pl. 1 and one Pl. 2	0	2	1
4. Both Pl. 2	22	14	8
5. One Pl. 2	8	11	2
6. Both Pl. 1	1	3	1
7. One Pl. 1	0	2	6
8. Both appendages on one side of the body	4	8	1
9. One Pl. 1 and one Pl. 2, of opposite sides	0	2	2
10. One ramus of Pl. 2	0	6	1
II. *Supernumerary structures:*			
1. Supernumerary Pl. 2	0	1	1
2. ,, Pl. 1	1	0	0
3. ,, Ramus of Pl. 2	0	4	2
4. ,, ,, ,, Pl. 1	13	10	8
III. *Homoeotic heteromorphs:*			
1. Thoracic limb on basis of Pl. 1	2	1	1
2. Ramus of Pl. 2 on Pl. 1	1	1	5
IV. *Fusion between two appendages:*			
1. Between the two members of a pair	6	1	0
2. Between the two limbs of one side	1	5	1
V. No normal structure absent (i.e. completely normal regenerations plus abnormalities other than deficiencies)	55	24	75
VI. Total number of survivors	117	147	107
VII. Mortality	112	168	156
VIII. Total number of operations	229	315	263

absence of one, two, or three limbs. Pl. 2 failed more frequently than Pl. 1, and the latter therefore rarely failed when Pl. 2 regenerated. In only one individual, in fact, did both Pl. 1 fail, while both Pl. 2 regenerated. This difference between Pls. 1 and 2 was observed in the other two series.

The most frequent supernumerary structure was an extra ramus (distal segment), developed on the medio-distal angle of the basal segment of Pl. 1. There is little doubt that this supernumerary ramus represents the normally suppressed endopodite of the appendage. Usually it failed to develop any specific differentiation, but in two individuals a typical thoracic limb was formed (though lacking one or two of the basal segments), while in a third, by contrast, it differentiated into a perfect Pl. 2 endopodite. In one individual one Pl. 1 was incompletely duplicated, with two rows of spines on the median aspect of the basal segment and two distal segments, one behind the other, presumably owing to subdivision of the blastema. Seven instances of fusion, of varying degree, between appendages were observed, six being transverse fusions between members of a pair. In the remaining example a complex Pl. 1 +Pl. 2 structure resulted.

One individual developed a supernumerary joint across the distal segment of *both* Pl. 1. The supernumerary (re-emergent) endopodite of Pl. 1 (p. 407) also appeared bilaterally more often (4/13 instances) than would be expected by chance, and the symmetry effect (p. 405) would seem to be a very general phenomenon (cf. p. 409).

Minor abnormalities of form, position, and orientation were common in this and in the other series. Some degree of rotation of a limb on its axis was not uncommon, and orientation is therefore not dictated solely by the main axes of the body. Both Pls. 1 and 2 were occasionally observed to regenerate the distal segment(s) directly on the body, the basal segment failing completely. Thus either proximal or distal segment may alone fail, a further manifestation of the all-or-none effect, here applied to individual limb-segments (cf. p. 409). While deficiencies within individual limbs were typically of the all-or-none type, however, occasionally an incompletely suppressed limb developed into a small styloid or flattened structure. Here, as in Amphibia (Needham, 1941 *a*), inhibition led to retardation, to a subnormal final size, and to incomplete differentiation.

In a number of experiments the copulatory styles (Text-fig. 2) were amputated. They regenerated normally provided a reasonable fraction persisted as a stump. Abnormalities of form and orientation occurred, but never any sign of heteromorphosis. Their normal form is too simple for extensive use in these experiments.

Of the twenty-seven surviving females of control experiments (Table 2), twenty-three showed normal regeneration, three failed to regenerate one of the Pl. 2 pair, and one failed to regenerate either of the pair. The contrast of this high incidence of regeneration to the behaviour in the male (pp. 405–7) is striking (see p. 417). This pair of appendages readily autotomize in the female, in contrast to those of the male (p. 405). On the other hand, they are attached to the body-wall by a very narrow base, in which the autotomy plane is located, so that the amount of the limb remaining to form a regeneration blastema is very small by comparison with the minimum necessary (p. 411) for regeneration in the male. Regeneration potency is considerably higher than in the male.

TABLE 2. *Analysis of Results of Experiments on Regeneration of Appendages (Pl. 2) of Second Abdominal Segment in female* Asellus aquaticus.

Same series as in Table 1.

Nature of the result	*Control series*	*Local-tissue series*	*Innervation series*
I. *Deficiency of:*			
1. One limb of pair	3	8	10
2. Both limbs	1	9	0
II. *Supernumerary limbs:*			
1. One supernumerary only . . .	0	2	2
2. Two supernumeraries to the same limb	0	1	0
III. Individuals with normal regeneration .	23	17	30
IV. Total number of survivors. . . .	27	37	42
V. Mortality	24	37	45
VI. Total number of operations . .	51	74	87

Histological examination of sections of this region of the body in males which failed to regenerate appendages showed the exo-skeleton and epidermis to be well healed over, though the site was recognized by the slight protuberance of the body-wall and by the presence of pycnotic nuclei. The peripheral nerves usually ended blindly in the body cavity near the site of the limb-base, without evidence of the neuromatous swelling often seen in such circumstances in Vertebrates (Highet, 1943). Inhibition of the limb would seem to be associated with inhibition of the peripheral nerve, whether as cause or effect, or merely incidentally. The possibility that suppression of regeneration may originate in the suppression of the peripheral nerve must be envisaged.

From the control experiments it may be concluded that while the Pl. 1 and Pl. 2 of the male do regenerate both accurately and rapidly under favourable conditions, nevertheless they have a conspicuously lower regeneration potency than thoracic limbs and also than the Pl. 2 pair of the female. This lower potency is most clearly reflected in the high incidence of complete failure to regenerate of whole limbs or of parts of limbs (Table 1). Regeneration potency would seem to be related to the degree of development of an autotomy mechanism and is in fact adaptive (cf. Needham, 1949*b*). Apart from the instances of re-emergence of the normally suppressed endopodite of Pl. 1, probably a completely independent phenomenon (p. 416), virtually all of the abnormalities were of the deficiency type. The results of the two experimental series (pp. 409–14) must therefore be analysed against this background of limited regeneration potency in the appendages.

The all-or-none and symmetry tendencies in the observed effects are particularly interesting and merit further consideration (pp. 415–16).

Experiments involving Post-amputational Treatment of Tissues at or near the Site of Regeneration (Local Tissue Series)

The wealth of atypical conditions observed in control experiments also characterized this series of experiments: indeed, the control experiment may be regarded as the least drastic type of the present series. The variety of induced effects renders analysis unreliable below the statistical level and in the first instance, therefore, all experiments of the series have been pooled (Tables 1, 2). The results are then seen to differ quantitatively and qualitatively from those of control experiments.

The incidence of abnormal conditions among survivors from the operation was higher (84 per cent. : 45 per cent.), the survival rate itself being, naturally, rather lower than in the control series. The higher incidence of atypical conditions was due almost entirely to the high incidence of complete failure of all four appendages, and the main effect of the treatments would therefore appear to be on the quantitative aspect of regeneration. The failure of three appendages was also relatively more frequent. The ratio of symmetrical to asymmetrical types of deficiency (75 : 42) is little different from that of the control series (p. 406), but if the individuals with total deficiency of all four limbs be omitted, then a much higher proportion of asymmetrical conditions is evident in the local tissue series. Deficiency of only one or two limbs, and of part only of a limb, was more common than in controls.

The somewhat lower incidence of a supernumerary ramus on Pl. 1 was perhaps due to the higher incidence of complete failure of Pl. 1, though the total number of Pl. 1 limbs regenerated was, in fact, almost the same in both series (control : 74; local tissues : 79). The ramus differentiated into a typical Pl. 2 endopodite in one individual and into a typical thoracic limb in another: in the others it failed to differentiate. Four instances of a supernumerary ramus on Pl. 2 were recorded in this series; this, like the converse picture (viz. of a deficiency of one ramus of Pl. 2), contrasts with the absence of such conditions in the control series, where the damage to the local tissues was less.

Also in contrast to the control series was a tendency towards a fusion between Pls. 1 and 2 of one side, rather than between the two members of a segmental pair. One individual developed a supernumerary complete Pl. 2.

Minor abnormalities of form were more common than in the control series but no further significant qualitative differences were detected.

The effects of specific procedures may now be considered (Table 3). Longitudinal incision of the stumps usually resulted in regression of tissue to the base of the incisions, followed by normal regeneration at that level. In the remaining individuals one ramus of one Pl. 2 failed (all-or-none effect in the ramus). The reason for this failure of a single ramus is not evident. The incidence of the condition in the whole series (see above) effectively depended on this sub-series which, otherwise, induced no specific abnormalities.

Maceration of the stumps after amputation caused a considerably higher incidence of failure of three and four limbs than any other treatment, and a

lower incidence of failure of two or one limbs (Table 3). No other condition was peculiar to this treatment, though the data indicate fewer supernumerary structures than in the control series. Maceration was followed by regression of the tissues of the stump (i.e. by removal of damaged tissue) and again, therefore, the regeneration blastema was formed, if at all, near the base of the limb.

TABLE 3. *Further Analysis of Results of Local-tissue Series in the Male* Asellus

	Type of Experiment in sub-series				
Nature of the result	*Stumps incised longitudinally*	*Stumps macerated*	*Ablation of body-wall between limb-bases*	*Embryonic tissue implanted*	*Chemical "treatment" of surface of stumps*
I. *Deficiency of:*					
1. All four limbs	0	19	13	14	12
2. Two Pl. 2 and one Pl. 1	0	6	7	4	0
3. One Pl. 2 and two Pl. 1	0	2	0	0	0
4. Both Pl. 2	0	2	6	3	3
5. One Pl. 2	0	0	5	4	2
6. Both Pl. 1	0	1	2	0	0
7. One Pl. 1	0	1	1	0	0
8. Both limbs on one side of body	0	1	3	3	1
9. Pl. 1 and Pl. 2, of opposite sides	0	0	2	0	0
10. One ramus of Pl. 2	3	0	3	0	0
II. *Supernumerary structures:*					
1. Complete Pl. 2 limb	0	0	1	0	0
2. ,, Pl. 1 ,,	0	0	0	0	0
3. One ramus of Pl. 2	0	0	3	0	1
4. One ramus of Pl. 1	0	2	4	2	2
III. *Homoeotic heteromorphs:*					
1. Thoracic limb on Pl. 1	0	1	0	0	0
2. Ramus of Pl. 2 on Pl. 1	0	0	0	1	0
IV. *Fusion:* (1) Between segmental pair	0	1	0	0	0
(2) Between Pl. 1 and 2 of same side	0	2	1	1	1
V. Individuals with complete regeneration	6	4	5	3	6
VI. Total number of survivors	9	39	45	31	23

Experiments (p. 404) in varying the level of amputation indicated that the high incidence of complete failure of limb-regeneration in the preceding sub-series was due to this proximal location of the blastema. Amputation at a different level on the two sides of the body showed that regeneration-rate decreased with the proximalward movement of the level of amputation, as in thoracic limbs (Needham, 1947). Complete suppression of regeneration, to be regarded as infinitely retarded regeneration (p. 414), was usual after total

amputation. At least one-sixth of the appendage must remain intact to ensure a high incidence of regeneration.

Destruction of the body-wall between the bases of the appendages did not induce fusion between appendages more frequently than in other sub-series. The most specific effect of the operation was an unusually high incidence of failure of one and two limbs, whereas that of three and four limbs, while also much higher than in control experiments, was lower than in the 'maceration' sub-series, owing to the less direct damage to the stumps. The results also imply a higher incidence of supernumerary structures than in the control series, probably due to partial subdivision of blastemata.

Implantation of embryonic tissue resulted in a high incidence of failure of three or four limbs, possibly due to mechanical damage at implantation. In addition, in about 30 per cent. of the animals treated, the implanted tissue continued its development, provided the donor had reached stage 3 (Needham, 1942*a*) of development. The interaction between donor and host tissues thenceforth appeared to be purely mechanical, and those host limbs which did regenerate were forced into abnormal positions. They were not affected qualitatively, and there was no fusion between regenerating and embryonic structures. Other aspects of the results of this sub-series merit consideration elsewhere.

The remaining experiments of the local-tissue series were preliminary investigations of the effect of acute treatment of the newly amputated surfaces with chemical agents. Data were too few for separate analysis of each. The general effect on regeneration appeared to be inhibitory.

The contrast between the results of operations on females of this local tissue series and those on control females is very striking. There was a high incidence of failure of one or of both limbs, and a relatively large number of supernumerary limbs, which were never observed in the controls. The number of limbs suppressed was a smaller fraction (26/74) of the possible total than for the males of this series (356/588), while the relative number of supernumerary whole limbs was higher than in the male series—an index of the higher regeneration potency of the female limbs. Heteromorphs were not observed and the very simple form of the limb (Text-fig. 3) was rarely abnormal. The failure of both limbs of the Pl. 2 pair occurred as frequently as that of one limb alone, another manifestation of the tendency towards symmetrical conditions (pp. 406, 409), and a particularly significant example, since the two limbs are more widely separated (Text-fig. 3) than those of the male (Text-fig. 2).

The main conclusion to be drawn from the local tissue series is that any damage to the stumps of these appendages tends to reduce the incidence of regeneration. There was a higher incidence of all deficiency conditions, including the failure of single segments of limbs, but particularly of failure of three or four of the set of appendages. Thus again (Needham, 1947) there is evidence that in this animal no substance with the properties of a 'wound hormone' is produced by damaged tissue at the site of the blastema. That destruction of tissue, by whatever means, should produce precisely the

opposite effect, namely, a reduction in regeneration potency, is probably due to the fact that regeneration is then initiated from a more proximal level of the limb where, as shown by varying the level of simple amputation, the intrinsic regeneration potency is lower.

Fusion between the two appendages of one side of the body was probably significantly due to this type of experiment. The close proximity of the bases of the limbs, combined with a low (proximal) level of regeneration, brings their respective regeneration fields within a critical distance for interaction. In this animal a further factor of importance is the customary withdrawal of the regeneration blastema and surrounding epidermis from the overlying exo-skeleton during the early stages of regeneration, so that the respective blastemata are not more isolated from each other than in soft-bodied types of animal.

The higher incidence of asymmetrical conditions than in control experiments is to be expected from the inevitable asymmetry of the experimental treatment. That the incidence of asymmetry was not even higher must be attributed in part to the all-or-none effect which tends to neutralize small differences between limbs and in part to the inherent tendency towards symmetry (p. 415), which would appear to operate even between widely separated members of the Pl. 2 pair of the female.

It is perhaps doubtful whether these forms of experimental treatment significantly affected the incidence of re-emergence of the endopodite of Pl. 1 in the male. The phenomenon would appear to depend on an inherent tendency which is independent of experimental treatment. The only two observed heteromorphs were due to this re-emergent ramus.

Qualitative effects due specifically to this type of experiment were therefore limited to fusions and supernumerary rami, and were relatively infrequent. Perhaps the most instructive results are those of the embryonic implantation subseries. Here limbs which did regenerate were usually quite normal, even though forced into abnormal position and orientation by a mass of developing embryonic tissue. The determination of the nature of an appendage would seem to be essentially contained within the appendage, to be largely unaffected by the disturbance of its spatial relations with other appendages of the set (or by the morphogenetic phenomena associated with the development of the graft). With such local determination of the nature of the regenerate frequent or extensive modification by experimental treatment is not to be anticipated. The blastema may occasionally be subdivided, partially or completely, when the isolated parts each regulate (p. 415) as wholes, or, again, two neighbouring blastemata may fuse, and the composite structure is simply a mosaic. Transposition of Pls. 1 and 2 was not observed, nor instances of heteromorphosis due specifically to 'local' treatments.

Experiments affecting the Nerve-supply to the Regenerates (*Innervation Series*)

The pooled results of these experiments are shown in Table 1 and are further analysed in Table 4. The mortality rate after unilateral ganglionectomy was probably not significantly different from that in the control series, but

after bilateral ganglionectomy and still more after nerve transection it was (p. 403) higher even than in the local-tissue series.

TABLE 4. *Further Analysis of Results of Innervation Series in the Male* Asellus.

Nature of the result	*Type of experiment in sub-series*		
	Nerve transection	*Ganglionectomy*	
		Bilateral	*Unilateral*
I. *Deficiency of:*			
1. All four limbs	5	1	0
2. Two Pl. 2 and one Pl. 1	4	0	0
3. One Pl. 2 and two Pl. 1	1	0	0
4. Both Pl. 2	8	0	0
5. One Pl. 2	1	1	0
6. Both Pl. 1	1	0	0
7. One Pl. 1	6	0	0
8. Both limbs on one side of body	0	1	0
9. One Pl. 1 and one Pl. 2 of opposite sides	1	1	0
10. One ramus of Pl. 2	1	0	0
II. *Supernumerary structures:*			
1. Pl. 2	1	0	0
2. Pl. 1	0	0	0
3. Ramus of Pl. 2	2	0	0
4. Ramus of Pl. 1	6	1	1
III. *Homoeotic heteromorphs:*			
1. Thoracic limb on basis of Pl. 1	1	0	0
2. Ramus of Pl. 2 on Pl. 1	5	0	0
IV. *Fusion between two limbs:*			
1. Between the two members of a pair	0	0	0
2. Between the two limbs of one side	1	0	0
V. No normal structure absent (i.e. completely normal regenerations plus abnormalities other than deficiencies)	28	37	20
VI. Total number of survivors	46	41	20
VII. Mortality	73	66	17
VIII. Total number of operations	119	107	37

Of the survivors in ganglionectomy experiments virtually all showed complete and normal regeneration. After unilateral operation the appendages of that side were usually retarded relative to their partners.

In the nerve-transection experiments, although abnormalities of regeneration among survivors were less frequent than in the control series, they were conspicuously more common than in the ganglionectomy sub-series. The ratio of deficiency conditions to other abnormalities (28/16) was considerably lower than in control (62/24) or in local-tissue series (123/23). The relative frequency of the various deficiency conditions also differed somewhat from that in the other series. Failure of a single Pl. 1 appendage, in particular, and of only one or two appendages, in general, was more common and the ratio of asymmetrical to symmetrical condition higher (17/15) than in local-tissue

(48/75) or control series (22/40). Supernumerary structures were possibly more frequent than in the other series, though total numbers are probably too small for a certain decision. Fusion between appendages was rare and this condition no doubt depends on 'local' disturbance. The incidence of asymmetrical deficiency conditions, and in particular of the failure of a single Pl. 1, was naturally more common after unilateral than after bilateral nerve transection. Limbs which did regenerate on the side of the operation were usually retarded relative to their partners.

In the female, where unilateral transection only was practised, bilateral failure of Pl. 2 was never observed (Table 2). This contrasts with the results of the local-tissue series in the female (Table 2), where failure of both limbs was as frequent as that of one only (p. 411). Among females, two supernumerary whole limbs were observed in a much smaller sample than that which yielded only one, among males. This sex-difference was noted also in the local-tissue series (p. 411).

The observed heteromorphic structures were again restricted to the re-emergent endopodite of Pl. 1. The number of heteromorphic Pl. 2 endopodites among them would seem to be significantly greater than in the other two series, though it is perhaps questionable, in view of the degree of insensitivity of the re-emergence to experimental treatment, whether this is to be attributed specifically to this type of experiment. Qualitative effects of denervation were indeed conspicuously lacking. Even quantitative effects, retardation, or complete suppression were rarer than in the other two series, though the frequency of unilateral retardation or failure following unilateral operation, in both sexes, clearly shows the same quantitative effect of denervation as in thoracic limbs (Needham, 1945). 'Local' effects were avoided in most experiments of the innervation series by amputating above the minimum level (p. 411), and it may therefore be concluded that the local tissues are more important than innervation even in the control of the quantitative aspect of regeneration. The extreme rarity even of quantitative (deficiency) conditions in the ganglionectomy sub-series was no doubt due to the inevitable rarity of complete destruction of the ganglia.

Discussion

From the results it seems clear that the local tissues are important for both the quality and for the quantitative aspects of regeneration, while the peripheral nerve-supply affects only the quantitative aspects. Temporary denervation of the stumps of these appendages (p. 412), as of thoracic appendages (Needham, 1945), leads to retardation of regeneration, and as in Amphibia (Schotté and Butler, 1944) permanent denervation results in complete suppression, the extreme of inhibition. On the other hand, the nerve-supply would seem to have no influence on the quality of the regenerate. If the peripheral nerve did in fact determine the nature of a regenerating limb, then at least a few instances would have been expected, particularly in the nerve-transaction sub-series, where errant re-innervation led to transposition of

Pls. 1 and 2 or to reversal of symmetry in one or more limbs (due to re-innervation by a nerve of the opposite side of the body).

The actual occurrence of such errant re-innervation remains to be demonstrated, but the experimental conditions were certainly ideal for such aberrations. Although the peripheral nerves of Crustacea do not lie completely free in the haemocoele, but are anchored by connective tissue, they are probably much less rigidly held, and less precisely guided in their regeneration by the neighbouring organs than in animals with closed circulation. They appear to be more easily and widely displaced by transection, and the cut ends probably retract more strongly (this is certainly true of the ventral nerve cord, which is often visible (see p. 403) in the living animal). In the present experiments, in addition, the nerves, lying in close proximity, were cut simultaneously at the same point and regenerated through a region where familiar histological landmarks had been destroyed. It seems scarcely conceivable that no single instance of such errant re-innervation occurred among survivors of the innervation series, and the absence of a single instance of limb-transposition is strong circumstantial evidence that, as in the ontogeny of Amphibia (Huxley and De Beer, 1934, p. 363), the peripheral nerve merely 'evokes' regeneration, the nature of the regenerate being determined locally (pp. 400–12), even down to details of orientation (pp. 407, 412).

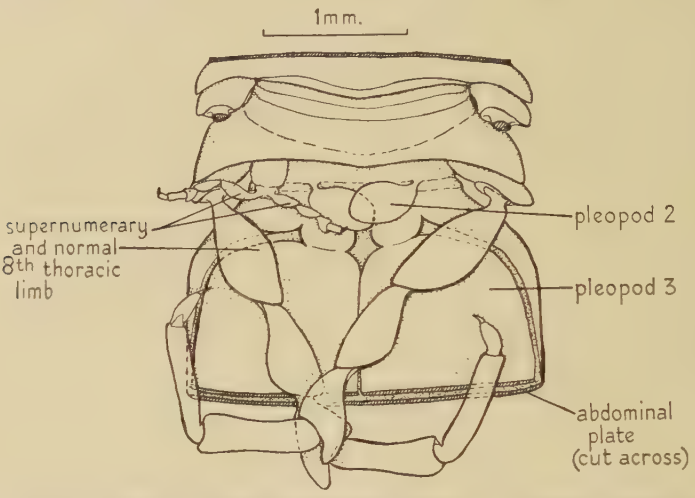
In view of the radical nature of some of the experimental treatments, the incidence of abnormalities, other than deficiency conditions and the re-emergent Pl. 1 endopodite, must be considered very low and the normal mechanism of determination is clearly proof against disturbance, to a high degree. Determination by the local tissues rather than by the nerve-supply is no doubt an essential part of this adaptive mechanism, minimizing the risk of topographical aberrations. The all-or-none effect (p. 405) may also be regarded as an adaptive mechanism, obviating minor quantitative abnormalities in regeneration, while the symmetry effect (p. 406) also helps to ensure normal regeneration.

The all-or-none effect would seem to imply a quantitative interaction between the parts of each unit: between the four limbs as a unit, between the two members of a pair to give the symmetrical type of deficiency condition, between the parts (segments) of individual limbs and even within individual segments of a limb (p. 407), in units of progressively smaller size. Within a unit some essential factor is presumably shared and the unit as a whole attains or fails to attain the threshold concentration of this factor necessary for complete regeneration. This 'quantisation' recalls that observed in fragments of explanted chick limbs buds (Murray, 1926). The proximity of the four limbs is no doubt important in determining the unity of the set, since partner limbs of the thoracic segments show considerable independence of each other (Needham, 1949*b*).

The symmetry effect is not merely quantitative, as in deficiency conditions, but also qualitative. The mirror-image property of bilateral symmetry is seen not only in normal development and regeneration but also in supernumerary limbs (Bateson, 1894). Text-fig. 3 shows an instance of this, of unknown cause, observed in a thoracic limb of *Asellus*. Each supernumerary appendage is the

mirror image of its nearest neighbour. This rule was observed by super-numerary structures in the present experiments. The quantitative aspect o symmetry-determination has received less attention than the qualitative aspect but in fact supernumerary limbs are usually equal in size (Bateson, l.c.).

Experimental treatment, especially where completely unilateral, tended t reduce the incidence of symmetrical conditions (pp. 409, 413), but there wa clearly a strong tendency towards symmetry, even in the face of these asym metrical disturbances. The interaction between the various limb fields, shown



TEXT-FIG. 3. Ventral view of thoracic-abdominal region of a female *Asellus aquaticus* to show (*a*) the normal form and position of the appendages of the second abdominal segment (setae omitted); (*b*) triplication of the right eighth thoracic appendage, with the usual symmetry interrelations (each limb the mirror-image of its nearest neighbour).

in these quantitative symmetry and all-or-none effects, and in the differentiation of the re-emergent Pl. 1 endopodite (p. 416), is an important aspect of field properties. Such an example of regulation as the adjustment of lens size to eye-cup size (Huxley and De Beer, 1934, p. 424) is no doubt a field-interaction phenomenon related to the present all-or-none effect, and indeed even the regulation of half blastomeres to give complete embryos (Huxley and De Beer, 1934, p. 98) may be the same phenomenon.

The incidence of re-emergence of the normally suppressed endopodite of Pl. 1 (p. 407) was seen to be virtually constant in the three series (pp. 409, 414) and insensitive to experimental treatment. There would, therefore, appear to be a quantitatively constant, inherent tendency for its re-emergence in regeneration. The re-emergence is further interesting in a number of respects.

It is surprising that when the ramus did show differentiation this bore no relation to the form it takes in those Isopods which normally possess the ramus (Zimmer, 1926, p. 717), but became either a typical thoracic limb or an equally good Pl. 2 endopod, as though the re-emergence were merely evoked, and not differentiated unless by the chance influence of neighbouring body-segments.

Records such as that of Young (1933) have shown that homoeotic structures need not necessarily be appendages typical of a more *posterior* segment of the body (Przibram, 1910). In the present example it was indifferently typical either of a more anterior or of a more posterior segment. Child (1941, p. 342) might explain this on the basis of individually variable ontogenetic changes in metabolic level, while Paulain (1938, p. 378) would explain it, perhaps more plausibly in this instance, as due to the overlapping by the thoracic-8 and the abdominal-2 limb fields of the first abdominal segment. It should be pointed out, however, that the eighth thoracic limb, in contrast to Pl. 2, is widely separated from Pl. 1 and that the example of Young (l.c.) cannot be explained by the postulation of overlapping neighbouring fields.

The re-emergence is another instance of atavism in regeneration (Korschelt, 1927, pp. 530–8). It is interesting also to the student of serial homology and indicates that (*a*) the normally missing ramus *is* the endopodite, and (*b*) that the thoracic limb corresponds to the endopodite of the primitive biramous appendage. In both respects orthodox views (Hansen, 1925, 1930) are vindicated.

The re-emergence has been observed also in *Asellus* (*Proasellus*, Rac.) *meridianus*, both in the field (Needham, 1941 *b*) and in laboratory experiments.

The relative ease with which regeneration may be retarded or suppressed in Pls. 1 and 2 of the male *Asellus* contrasts sharply with the behaviour of the thoracic appendages of this animal (p. 405). A possible explanation might lie in the great morphological complexity (p. 403) of the former. The lower frequency of failure of Pl. 1 than of Pl. 2 (p. 406) might then be due to the less complex form of Pl. 1, and the rarer failure of Pl. 2 in the female than in the male might similarly be explained. On the other hand it is perhaps questionable that the form even of Pl. 2 of the male is more complex than that of thoracic limbs which, by contrast, regenerate (p. 405) with great fidelity, and the more plausible explanation would seem to be (p. 405) that Pls. 1 and 2 are not normally subject to regeneration and have an imperfect regeneration-mechanism. The absence of an autotomy plane at the base of these limbs (p. 405) is part of this imperfection. These appendages of the male have rarely been observed damaged or regenerating among animals from the field, and no doubt they are normally not subject to amputation. On the other hand the Pl. 2 appendages of the female have the power of autotomy (p. 407), and are more frequently observed to regenerate in the field. This would seem to provide further evidence that the power of regeneration in animals is adaptive and that the perfection of the mechanism is related to its adaptive value (Needham, 1949 *b*), and is not merely a persisting pristine property of living organisms. It is further interesting to find such a contrast in power of regeneration between different limbs of the same animal. The reason for the difference in potency between Pls. 1 and 2 of the male is not evident.

A factor in the induction of abnormalities in these regenerates is undoubtedly the proximity of the four limbs. Fusion of two blastemata, whether transversely or longitudinally to the body (pp. 407, 409), could not otherwise occur. Occasionally a typical Pl. 2 exopodite developed on the posterior face of

Pl. 1 (a condition unrelated to the homoeotic differentiation of a Pl. 2 endopodite from the re-emergent ramus of Pl. 1) and such apparent examples of supernumerary structures may well be due to the partial fusion of the Pl. 2 blastema with that of Pl. 1, the remainder of the Pl. 2 blastema, nevertheless, developing into a complete Pl. 2 appendage in conformity with the all-or-none phenomenon. Attempts to induce fusion (Table 3), however, gave a high proportion of supernumerary structures (p. 411).

It is once more (cf. Needham, 1947) evident that in this animal increased damage to tissues does not accelerate but rather inhibits regeneration (p. 411), and that here there is no 'wound hormone' with the function of a regeneration-stimulant. Further, from a comparison between local-tissue and control series it would seem that an irregular surface of amputation (p. 410) does not specifically induce duplication or higher degrees of multiplication of the regenerate, as it does in Planaria (Lus, 1924) and possibly in Amphibia and other groups (Huxley and De Beer, l.c., p. 299), and indeed in other Arthropods (Przibram, 1921). The damaged tissues in this Crustacean normally regress to form a relatively plane surface for regeneration.

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The Mesoglea and Muscle-fibres of *Chlorohydra viridissima*

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With two Plates

Summary

The structure and relations of the mesoglea, muscle-fibres, and cell layers were examined by several histological techniques.

Evidence is given that the muscle-fibres represent specializations of the mesogleal substance, and they are therefore referred to as mesogleal fibres.

The longitudinal fibrous system is regularly demonstrable in the body of the animal and the circular system in its tentacles. The circular system in the body cannot regularly be shown and is variable in its appearance.

The evidence from the study of the fibres, and of the mesoglea of the pedal disk, leads to the suggestion that some at least of the fibres are labile structures, 'crystallizing' from the mesoglea according to the shape and movements of the animal.

Introduction

THE movements of hydra are usually considered to depend upon the contraction of muscle-fibres lying in or upon the mesoglea. Two systems of fibres act antagonistically: there is said to be an outer layer in which the fibres are longitudinal, and an inner layer in which they are transverse, encircling the body-wall. The outer system of fibres is thought to be intimately related to those cells in the ectoderm known as musculo-epithelial cells. The inner system is connected to the bases of the endoderm cells, though the latter are not usually described as musculo-epithelial, since the relation between cells and fibres seems less intimate.

It is not proposed here to review the extensive literature on the histology and cytology of hydra; a recent paper by Mueller (1950) gives key references, and makes some additions to our knowledge. A careful study of the literature leaves the reader still uncertain of the general micro-anatomy of the contractile system in any one species of the animal. This is in part due to the fact that the techniques employed have not been such as to reveal the relations in the contractile system as a whole: microtome sections of hydra are particularly difficult to interpret, and the technique of maceration reveals only the relations within small fragments of the animal, and is unsuitable for demonstrating the total organization.

Most teachers of zoology are familiar with the appearances seen in macerated hydra preparations, and they are admirably illustrated by Goodrich (1942). One feels, nevertheless, that maceration is an imperfect method of revealing

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the relations of an extended fibrous system. Furthermore, since many macera-tion processes are no more than controlled autolysis, or employ reagents in-tended to destroy those parts of a tissue which bind its cells together, they may well give deceptive appearances.

It was thought desirable, therefore, to study the relations of the contractile system only in material which had been properly fixed, and which was ex-amined in whole preparations and in sections.

Material and Methods

The investigation was confined to the green hydra, *Chlorohydra viridissima*. Specimens were fixed at all stages between full elongation and contraction, by the following technique. A group of some 30 individuals was allowed to settle down in a glass vessel containing about 25 ml. of pond water. After about half an hour 4 drops of pure chloroform were added to the water, and the vessel covered with a glass lid. During the next 15–45 minutes many of the animals became inactive, some in a fully extended condition, others in varying degrees of contraction. When it was found that some of the extended individuals were so far anaesthetized as not to react to mechanical stimulation, 100 ml. of the required fixative were poured into the water. Some of the extended animals immediately contracted, but others remained extended. Since the fixative was diluted by this procedure the whole of the fluid was withdrawn after 2 or 3 minutes, when the animals were dead, and a new quantity of fixative poured in. Individuals showing various degrees of elongation were selected with a pipette and the surplus rejected.

Histological Techniques

A. *Flemming Material*

Fixative: 0·8 per cent. NaCl in distilled water . . . . 30 ml.
1 per cent. chromic acid in 0·8 per cent. NaCl . . 30 ml.
1 per cent. osmium tetroxide in distilled water . . 16 ml.
Pure acetic acid . . . . . 5 drops

Duration of fixation: 24 hours.
Wash in running water: 3 hours.
Embedding in wax through chloroform.
Sections at 4μ, 7μ, and 12μ.
Stain: 2 per cent. iron alum 24 hours; rinse; Heidenhain's haematoxylin 24 hours; differentiate in 2 per cent. alum until black fibrils distinguishable. All procedures carried out at room temperature.

B. *Del Rio Hortega's 'Connectivo' Technique*

This method was designed by its author for the demonstration of the finest fibrils of mammalian connective tissue. It is normally applied to frozen sections but my procedure with hydra was to stain the animals entire, treating each individual as if it were a frozen section. Animals were carried through in groups of about 10, in glass centrifuge tubes, the changing of fluids being facilitated by the use of a hand centrifuge and a fine pipette attached to a water-suction pump.

Solutions required:

0·2 per cent. potassium permanganate in distilled water.
10 per cent. oxalic acid in distilled water.
Strong ammonia.
2 per cent. $AgNO_3$ in distilled water; add 2·5 ml. pure pyridine to each 100 ml. shortly before use.
10 per cent. $AgNO_3$ in distilled water.
Formalin: 25 ml. of 40 per cent. formaldehyde in 75 ml. distilled water.
5 per cent. Na_2CO_3 in distilled water.
0·2 per cent. gold chloride in distilled water.
5 per cent. sodium thiosulphate in distilled water.

'Weak silver sodium carbonate': To 10 ml. of 10 per cent. $AgNO_3$ add 40 ml. of 5 per cent. Na_2CO_3. Then immediately add strong ammonia drop by drop, agitating continually, until the solution has reached a point when it is just turbid. *It is useless if it is clear.* Dilute to 200 ml. with distilled water. Filter. Use within 24 hours. Place the solution in a paraffin oven at about 50° C. so that it will have reached this temperature when required for use; immediately before use add 5 drops of pyridine to each 20 ml. of solution.

Procedure.

1. Fixative: 40 per cent. formaldehyde . . . . . 10 ml.
   Pond water . . . . . 90 ml.
2. Duration of fixation: 12 hours to 3 days.
3. Specimens taken from the fixative and treated with distilled water containing 1 per cent. strong ammonia: 5 minutes.
4. Wash in distilled water: 5 minutes.
5. Treat with 0·2 per cent. potassium permanganate. After 60 seconds' treatment begin to remove specimens and carry them to stage 6; remove them at short intervals so that the length of permanganate treatment varies between 60 seconds and $2\frac{1}{2}$ minutes for different individuals.
6. Wash in distilled water, three changes, 5 minutes.
7. 10 per cent. oxalic acid, 3 minutes.
8. Ammoniacal water, 12 drops strong ammonia to 35 ml. distilled water, two changes: 10 minutes.
9. Distilled water, two changes: 5 minutes.
10. Place in 2 per cent. silver nitrate containing 2·5 per cent. pyridine, previously brought to 50° C.: 30 minutes at 50° C.
11. Transfer to 'weak silver sodium carbonate', with the pyridine added: 20 minutes at 50° C.
12. Rinse for 10 seconds in distilled water.
13. Transfer to the formalin solution, changing it frequently and rapidly as it becomes cloudy: 15 minutes.
14. Wash in several changes of distilled water, duration not less than 10 minutes.
15. Tone in 0·2 per cent. gold chloride: 10 minutes.
16. 5 per cent. sodium thiosulphate: 5 minutes.
17. Wash in distilled water; dehydrate.

Some specimens are mounted whole in balsam, under a supported cover-slip; the most useful preparations, however, are obtained by embedding the stained specimens in wax, cutting sections at 20μ, and covering without counterstain.

This elaborate procedure gives variable results, but successful preparations yield a brilliant demonstration of the muscle-fibres.

C. *Phosphotungstic Haematoxylin*

Specimens fixed in Zenker's fluid were sectioned at 8μ and stained with either Lendrum's, Mallory's, or Russell's variant of the phosphotungstic-haematoxylin stain. The most useful results were given by following the procedure of Russell (1939). The muscle-fibres of hydra are stained blue.

D. *Linder's Technique* (1949)

This was too recently published to have been extensively used in my work, but it gives an excellent deep-blue stain to the mesogleal fibres, when applied to sections at 7μ of material fixed in formaldehyde/mercuric chloride, as recommended by its inventor. Linder's technique may well prove the simplest and most desirable method for the demonstration of the mesogleal fibres in permanent preparations for class use. This method was devised so as to achieve the same results by staining as are given by silver impregnation in the 'connectivo' technique of del Rio Hortega.

Results

The Mesoglea of the Pedal Disk

The Hortega preparations show a variety of appearances in the pedal disk region which can best be interpreted by assuming that the mesoglea has some fluid properties. The layer in question is much thicker in this zone than elsewhere in the column or tentacles, and is deeply stained by the silver technique. The mesogleal substance so demonstrated shows a variety of appearances, so that its form is never exactly the same in any two specimens. It seems to be a 'pool' of material which can appear in various shapes; Pl. I, figs. 1 and 3 show two of these variants. The pool is 'perforated' in the central area of the disk, at a point apparently corresponding with the aboral pore (Pl. I, figs. 2 and 3).

The Longitudinal Muscle-fibres

Longitudinal fibres are seen clearly in the Hortega preparations (Pl. I, figs. 7 and 8), in the Flemming material (Pl. I, fig. 4; Pl. II, figs. 11, 12, 13, 17), and in the Linder preparations. They lie, of course, between ectoderm and endoderm, and are undoubtedly the longitudinal muscle-fibres of other authors. Several reasons lead me to believe, however, that it is unwise to regard them as independent structural elements resting on or in a distinct mesogleal substance, and I shall therefore refer to them as mesogleal fibres.

As will be seen from the figures, a longitudinal section of a Hortega pre-

paration, or a fully differentiated haematoxylin section, shows the mesogleal fibres as distinct elements, with the intervening mesogleal substance unstained. But, on the other hand, every transverse section of a Hortega preparation shows the mesoglea as a continuous ring, and not as a circle of separate dots, representing the individual fibres. It will be recollected that the Hortega material is stained before sectioning, and transverse and longitudinal sections should therefore be directly comparable. I interpret this apparent contradiction in the appearances in the following way.

It is generally recognized that in silver-impregnated tissues the demonstration of specific structures is not due to a specific deposition of silver on those structures alone; on the contrary, silver is deposited in or on every element of the tissue. Those parts of it which are 'specifically stained' are those on which the silver is more densely deposited, or deposited in a different physical state; this makes the element concerned appear darker against a lighter background. Thus in a longitudinal section of a silver-impregnated hydra the mesoglea between the fibres appears unstained because it is transmitting more light than the 'specifically stained' fibres. Now at a point in the body-wall where the mesoglea has a thickness of about $1{\cdot}5\mu$ in transverse section, the fibres seen in longitudinal section are less than $0{\cdot}5\mu$ in diameter. When examining a transverse section one is looking through a layer of mesoglea with fibres that is as thick as the section: that is, many times thicker than the mesoglea as seen in a tangential longitudinal section. The uniform staining seen in the transverse sections is thus explicable. This interpretation leads to the conclusion that the sharp distinction between fibres and mesoglea is, to some extent, a deceptive appearance. It also accounts for the fact that, in whole mounts of Hortega preparations of contracted animals, the fibres can only be resolved microscopically at the edge of the preparation and not at those points where the light is passing through the full thickness of the animal's body.

The appearances in the haematoxylin preparations may be interpreted in a similar way. The sharp picture of distinct fibres is obtained by differentiation up to a point at which the appearances are 'satisfactory' to the observer looking down the staining microscope; that is to say, to a point when the haematoxylin has been sufficiently removed from the non-fibrous mesoglea to leave it colourless in contrast with the fibres.

Now staining by Linder's method does not involve a differentiation, and in these preparations the non-fibrous mesoglea is stained pale blue, while the fibres are dark blue in colour; here again, in transverse section, the fibres are indistinguishable in the continuous dark-blue mesogleal ring.

Equally suggestive are the appearances in Hortega preparations, in the region of the mesogleal 'pool' in the pedal disk. Pl. I, figs. 1 and 2 are of contiguous sections at 20μ through this region of a silver impregnated specimen. The dark-blue, homogeneously stained, pool of substance (Pl. I, fig. 1) is continuous with the substance of the longitudinal mesogleal fibres (Pl. I, figs. 1 and 2). Similarly a fibrous appearance can be seen in the section illustrated in Pl. I, fig. 3. The longitudinal fibres 'radiate' from the pedal disk region

up throughout the mesoglea in the column (Pl. II, fig. 11, haematoxylin preparation) and would seem, from the silver preparations, to be of the same material as the semi-fluid mesoglea of the disk.

It is suggestive at this point to consider the purposes for which the Hortega and Linder techniques were intended by their authors. These are, first, the staining of the finest fibrils of vertebrate connective tissue—the reticular fibres—and, second, the demonstration of the basement membrane of the mammalian renal glomerulus, which is a difficult feat of histological technique. Now it is supposed on other grounds that the glomerular basement membrane is a continuous sheet of substance, and it is perhaps not too remote an analogy to suggest that both glomerular membrane and mesoglea have the common feature of a regional fibrous organization within a continuous layer. Both layers are thus made visible, under certain optical conditions, by histological methods which pick out the more finely organized fibres.

The tentative conclusion from these studies of the longitudinal fibres and the mesoglea is that the fibres are formed of mesogleal substance which possesses a fibrous organization at the micro-anatomical level. Evidence of the fluid properties of the mesoglea suggests that the fibrous system may be of a labile kind, and that the appearances seen in microscopical preparations may represent the condition of a changing system as fixed at the moment of death.

Reliable estimates of the length of longitudinal fibres can only be made by the study of those sections which include a considerable stretch of the mesoglea, as in Pl. I, fig. 8, and Pl. II, figs. 12 and 13. Such preparations are very much more readily obtained from animals fixed in extension; tangential sections of the fully contracted animal, which is almost spherical, each contain only a narrow strip of mesoglea. So, unfortunately, it is impossible to compare the lengths of the fibres in contraction with those in extension.

Inspection of the figures mentioned above will show that the maximum length of single fibres, as visible in the one focal plane photographed, is of the order of 80μ. Up-and-down focusing on these thick sections, however, shows much greater maxima. I have seen many fibres which appeared to me to be at least 250μ long (0·25 mm.) in these moderately extended animals, and this is a cautious estimate; some may be much longer. There are as well, it seems, many shorter fibres, but this statement also must be made with the proviso that many fibres will appear to be shorter than they really are because they pass out of the plane of section. The examination of whole silver-impregnated individuals is liable to lead to misinterpretation, as already explained; those preparations would lead one to suspect that many of the longitudinal fibres run along the whole length of the column, from pedal disk to oral cone.

Examination of Pl. I, figs. 7 and 8 (which are of the same object), and Pl. II, figs. 12 and 13, will give a fair picture of the arrangement and relations of the fibres. They sometimes, but rather rarely, seem definitely to branch; apparent branches are more usually points of contact between two fibres, and a discontinuity can be seen. Definite folding of the fibres is unusual, but can be seen in Pl. II, fig. 12.

The Circular (Transverse) Mesogleal Fibres

It is clear that the circular fibres are in some respect different in quality from those of the longitudinal system since they cannot regularly be demonstrated in preparations in which the longitudinal fibres are sharply stained. That this is not due to a failure or vagary of the technical methods is indicated by the fact that in the hydroid *Tubularia* both circular and longitudinal fibres can readily be demonstrated in the same preparations by the haematoxylin technique here employed on *Chlorohydra* (D. E. Moorhouse, *personal communication*).

Furthermore, in those preparations in which transverse fibres are to be seen there is great variation in their form and number. Sometimes they appear as faint striations running across the preparations, more numerous than the longitudinal fibres (Pl. II, fig. 13). On other occasions, usually in contracted animals, clear cross-fibres run round a proportion of the circumference of the body; these are as large in diameter as the longitudinal fibres, but less numerous (Pl. I, fig. 5).

In animals in which there was a 'waist' in the column at the time of fixation, or in those which were bent over to one side at that time, there often appear very clear and numerous transverse fibres, but these appear to be connected with the longitudinals; they form cross-bars joining two or three longitudinal fibres together, and they often appear along kinks in the mesoglea due to folding of the body-wall. The clearest of all transverse fibres, which also appear to be continuous with the longitudinals, are seen in the mesoglea below ripe gonads, where fibres orientated in both directions form a dense network of thick component strands; this is illustrated in Pl. II, figs. 9 and 10, which are of contiguous silver-stained sections at 20μ showing the mesoglea in such a situation. This is undoubtedly a specialization in relation to gonad formation, for there are extensions of the mesogleal fibres into the gonad tissue.

I have had great difficulty in interpreting these varying appearances, particularly in reconciling them with the accepted view of the distribution of the transverse fibres and their relations to the endoderm cells (Mueller, 1950, points out that there are disagreements in the literature on this matter). Pl. II, fig. 17, gives a helpful indication. It is of a longitudinal section of the body-wall, haematoxylin stained, with ectoderm above and endoderm below. Since the section was 7μ thick several distinct longitudinal fibres are seen, coursing from left to right. It will be noticed that there are points of thickening, or of deeper staining, in the longitudinal fibres, and that these coincide with the points at which the fine cell-walls of the endoderm cells make contact with the mesoglea.

A provisional conclusion from these observations is that transverse mesogleal fibres are not anatomically separate from the longitudinal fibres and from the mesogleal substance. Their sharper appearance in the region of a waist or in contracted animals suggests that the mesoglea undergoes changes with the changes in form of the animal, and that these may result in a reversible 'crystallization' of transverse fibres. There is evidence of transverse fibrillation

at the points in the mesoglea where the cell-walls of adjacent endoderm cells make contact with each other and with the mesoglea. The appearances seen in macerated preparations will thus be variable, and the 'transverse muscle-fibres' will be detached portions of mesogleal substance with a transverse orientation.

The Fibrous System in the Tentacles and Oral Cone

In the tentacles we see a reverse appearance from that in the column, for the major fibrous system is circular, and the longitudinal system unclear and variable. The circular fibres are sometimes of the same order of diameter as the longitudinals of the column (Pl. II, fig. 14); in other specimens they form thicker rings, particularly towards the tip, as in Pl. II, fig. 15, a photograph of a Hortega whole mount. Fine longitudinal fibrils are visible near the base of the tentacle, but rarely so towards its tip, and in greatly elongated tentacles the transverse fibrils also 'disappear' in so far as they are no longer made visible by staining.

Some of the longitudinal fibres of the column continue up into the region of insertion of the tentacles, but the greater part of the system converges into the oral cone, where the fibres are more densely concentrated because of the decreased circumference of the body. The fibres converge to the mouth just as they do to the pedal disk. I have not been able to observe circular fibres in the oral region.

The Relations between the Fibres and the Cells

The cells which secrete and maintain the mesoglea must be situated in either ectoderm or endoderm or both, for none are found within it.

A special relationship exists between the mesogleal fibres and the ectoderm cells of the pedal disk. From the mesogleal 'pool' in this region there arise not only the longitudinal fibres which pass up the column in an oral direction, but also short fibres which turn aborally and pass into the ectoderm layer. These fibres are best seen in the Flemming-haematoxylin preparations (Pl. I, fig. 6). They are very numerous, more than one per cell, and can often be seen to pass as far as to the outer free surface of the cells. The pedal disk cells are not sharply demarcated from each other by a distinct cell-wall, so it is usually impossible to be dogmatic as to whether the fibrils lie in an intracellular position or are applied to the outer surface of the cytoplasm (Pl. I, fig. 6). Sometimes, however, a fibre can be seen bending round the nucleus of a pedal cell, as if it had an intracellular position.

A different sort of relationship exists between the longitudinal fibres and the ectoderm cells of the column, as seen in Pl. I, fig. 4. This is a photograph of a longitudinal Flemming-haematoxylin section, in which the ectoderm and part of the mesoglea have conveniently become detached from the endoderm during sectioning. It is clear from this and from many other preparations that any given fibre has an equally intimate relationship with several ectoderm cells. The relations between cells and mesogleal fibres are much like those

between fibroblasts and the fibres they secrete. In my preparations it is impossible to distinguish a definite boundary between the cytoplasm and the underlying fibre nor, often, between the cytoplasms of adjacent cells. If we accept the view that the mesoglea is a continuous sheet in which the fibres are 'crystallized' then the cytoplasm of the ectoderm cells has an intimate relation with the whole mesogleal system.

At many points in the ectoderm the mesogleal fibres seem to pass outwards into the cellular layer, reaching almost to its outer surface (Pl. II, fig. 16). The appearance given in such material is that of the 'sensory cells' of some investigators; the condition is in general similar to that in the pedal disk, but the fibres which pass outward in this way in the column are far fewer in number in relation to the number of ectoderm cells concerned.

As is well known, the endoderm cells of the column are box-like in that they have clear cell boundaries but, near the mesoglea, contain little cytoplasm other than that which forms the cell border. They appear, therefore, to lie on the mesoglea, but their relationship with it is very intimate, since many preparations show a continuity of stained material between the endoderm cell-walls and the mesogleal fibres. In the endodermal pharyngeal folds, in the oral cone, this relationship between mesogleal fibres and the long side walls of the cells is more clearly and certainly demonstrated. For mesogleal fibres pass inwards between the endoderm cells and extend as far as the inner free border of the endoderm. These fibres appear to be continuous with those of the longitudinal system, and are of approximately the same diameter. This intimacy between endoderm cells and longitudinal fibres is evidence that the longitudinal system cannot be regarded as solely associated with the ectoderm.

At the bases of the tentacles inward-turning fibres are equally clearly seen, and, applied to the surface of the endoderm cells, they often appear to form a cross-connexion between different quadrants of the mesoglea.

Discussion

The presentation of the observations described in this paper has already involved a good deal of speculation, and it would be unjustifiable to carry it much farther. It has recently been made clear that the 'classical' accounts of the micro-anatomy of hydra provide an insufficient basis for a full correlation of its behaviour with the anatomy of its conducting and contractile mechanisms (Pantin, 1950). The present work was begun in the expectation that a straightforward account of the anatomy of the 'muscle-fibre' system would help to bring about this correlation. But this has not proved to be the case.

The mechanics of the behaviour of hydra, with its extremely thin mesoglea and enormous powers of elongation, are likely to show differences from the condition in such higher forms as the anemones of which we now have a good deal of information. It is possible that the possession of a mesoglea with a fibre structure of varying stability, elastic or even contractile, is a part of the basis of these differences.

It was hoped to include a study of the nervous system in the present investigation, but this proved to be impossible. The application of a variety of methods to the column led to an increasing lack of confidence in the identification of particular cells and their processes as being exclusively nervous in character. We have still not disproved the old speculation that the structural basis of hydra's behaviour lies in 'neuromuscular' cells (Kleinenberg, 1872).

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EXPLANATION OF PLATES

All the photographs are of longitudinal sections of *Chlorohydra viridissima*, except Fig. 15, which is of a whole preparation of the same animal.

Plate I

Figs. 1 and 2. Hortega's method. Contiguous sections of the pedal disk at 20μ.

Fig. 3. Hortega's method. Middle region of the pedal disk showing perforation of the mesogleal pool in the region of the aboral pore. 20μ.

Fig. 4. Flemming: haematoxylin. Ectoderm of the column, with underlying mesogleal fibres. 7μ.

Fig. 5. Hortega's method. Tangential section showing a sheet of mesoglea seen from within. Endoderm cells are in focus to the right. 20μ.

Fig. 6. Flemming: haematoxylin. Portion of the pedal disk; ectoderm above, endoderm with zoochlorellae below. 7μ.

Fig. 7. Hortega's method. Tangential section. Detail of longitudinal mesogleal fibres. 20μ.

Fig. 8. Hortega's method. Tangential section showing a long sheet of mesoglea. 20μ.

Magnification: the ruled line represents 40μ for Figs. 1, 2, 3, 5, and 8; 20μ for Figs. 4, 6, and 7.

Plate II

Figs. 9 and 10. Hortega's method. Contiguous tangential sections showing the mesogleal fibres below an ovary. 20μ.

Fig. 11. Flemming: haematoxylin. Basal region of the animal showing longitudinal fibres radiating from the pedal disk. 7μ.

Fig. 12. Flemming: haematoxylin. Detail of longitudinal fibres. Ectoderm cells in focus above, zoochlorellae to the right. 7μ.

Fig. 13. Flemming: haematoxylin. Longitudinal fibres, and a faint close transverse fibrillation. 7μ.

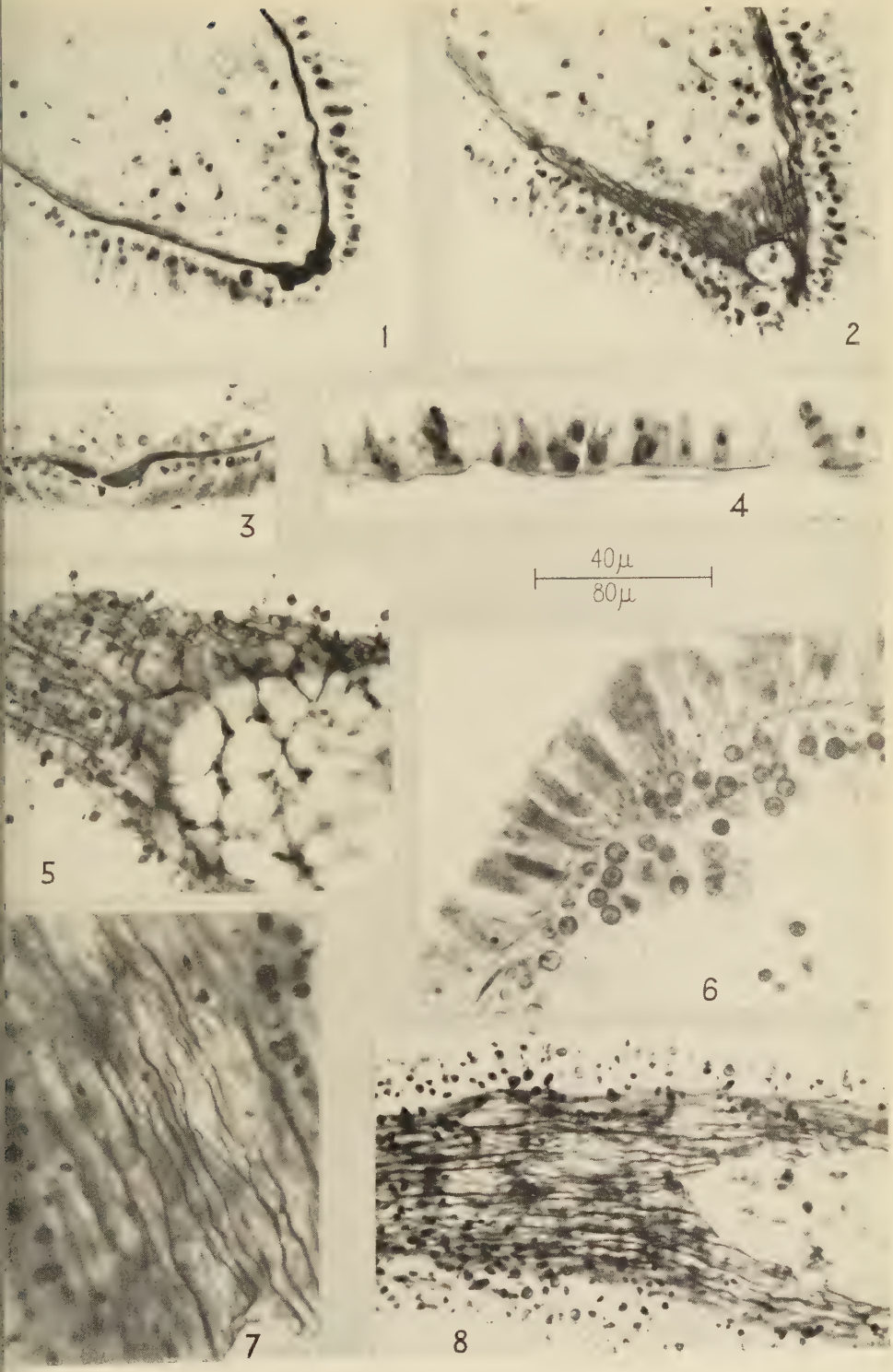
Fig. 14. Hortega's method. Distal half of a tentacle in longitudinal section. 20μ.

Fig. 15. Hortega's method, whole mount. End of tentacle.

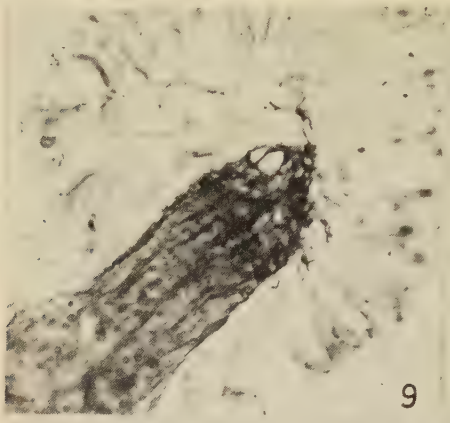
Fig. 16. Flemming: haematoxylin. Detail of ectoderm and mesogleal fibres. 7μ.

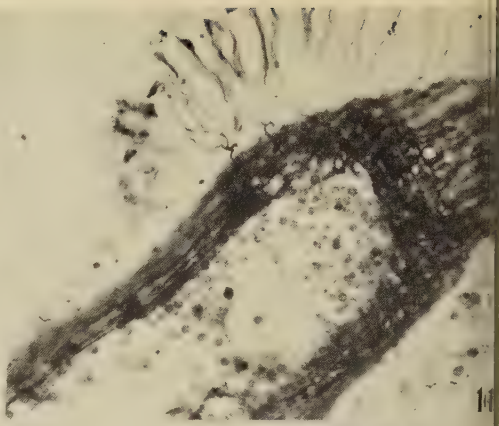
Fig. 17. Flemming: haematoxylin. Ectoderm above, endoderm below. 7μ.

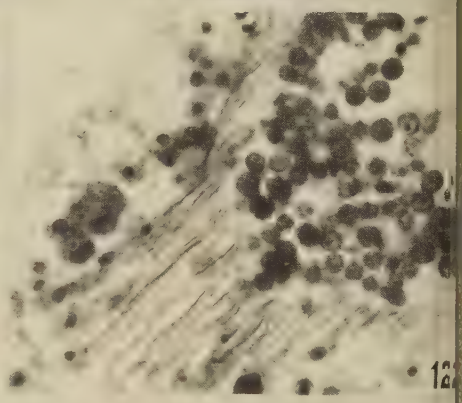
Magnification: the ruled line represents 40μ for Figs. 9, 10, 11, 14, 15, and 17; 20μ for Figs. 12, 13, and 16.



W. HOLMES—PLATE I







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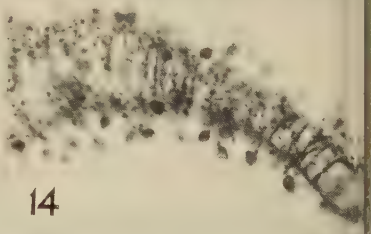

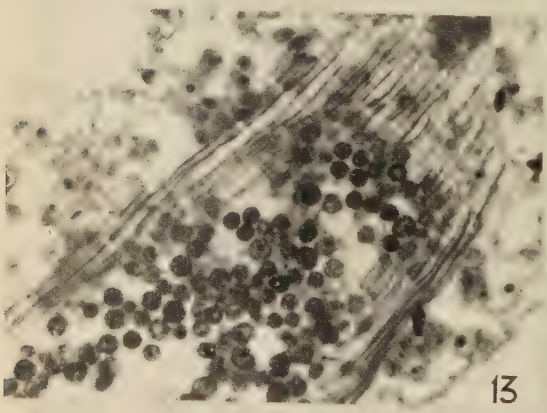

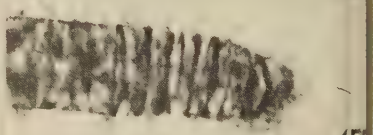

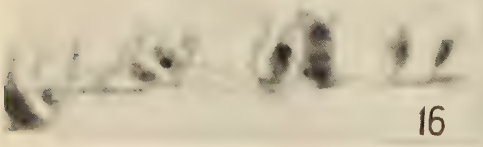

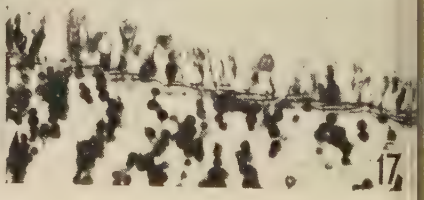

W. HOLMES—PLATE II

The Respiratory Mechanisms of Some Insect Eggs

By V. B. WIGGLESWORTH AND J. W. L. BEAMENT

(*From the Agricultural Research Council Unit of Insect Physiology, Department of Zoology, Cambridge*)

With one Plate

SUMMARY

By the use of the cobalt sulphide injection technique the distribution of air in the shell of a number of insect eggs has been studied. Air is usually confined to an inner layer of porous protein, connected with the atmosphere through pores of varying type which are likewise filled with spongy material.

In *Rhodnius* the 'resistant protein layer' which lines the shell is the porous structure and the 'pseudomicropyles' connect this layer to the exterior. The arrangement in *Cimex* is similar. In *Oncopeltus* the spongy walls of the 'sperm cups' convey air to a porous inner layer. After laying, the lumen of each cup (the micropylar canal) is occluded with solid cement.

In *Dixippus* the so-called 'micropyle' in the 'scar' of the egg is the respiratory pore. It is filled with spongy protein containing air and conducts the air to the spongy inner layer of the endochorion. As the egg develops and its contents are reduced in volume, free air collects between the two layers of the endochorion in the region of the pore.

In *Blattella* an elaborate stigmatic apparatus which is moulded in the crista of the oötheca conveys air to a spongy process at the upper pole of the egg and so to a thin porous air-filled layer which lines the chorion.

In *Bombyx* and *Ephestia* a thin porous inner layer of the chorion containing air communicates with the exterior through scattered pores containing air-filled spongy material.

In the eggs of Diptera the chorion consists of tapering columns with spongy walls which unite the cement-covered outer layer to a spongy inner layer containing air. The horns on the *Drosophila* egg and the dorsal folds on the *Calliphora* egg provide respiratory outlets for this system. The spaces between the columns contain liquid in *Calliphora* and *Drosophila*; in *Syrphus* these spaces are greatly enlarged and contain air.

The spongy layers may become filled with air in eggs which are still bathed in fluid in the oviduct, or in which water is present in adjacent parts of the shell. The mechanism of filling is discussed.

In the case of *Rhodnius* there is quantitative evidence that the system will provide for the respiratory needs of the egg.

IT has been shown experimentally by Tuft (1950) that the entry of oxygen into the eggs of *Rhodnius prolixus* takes place almost wholly in the region of the cap. Knowing the rate of oxygen consumption in the egg, and the approximate speed of diffusion of oxygen through living tissues, Tuft proved by calculation that oxygen could not diffuse from the cap into the more remote portions of the egg sufficiently rapidly to meet the oxygen requirements—unless there is a film of air extending around the inside of the shell. Finally, he was able to show that if the cap of the egg were touched with a drop of oil,

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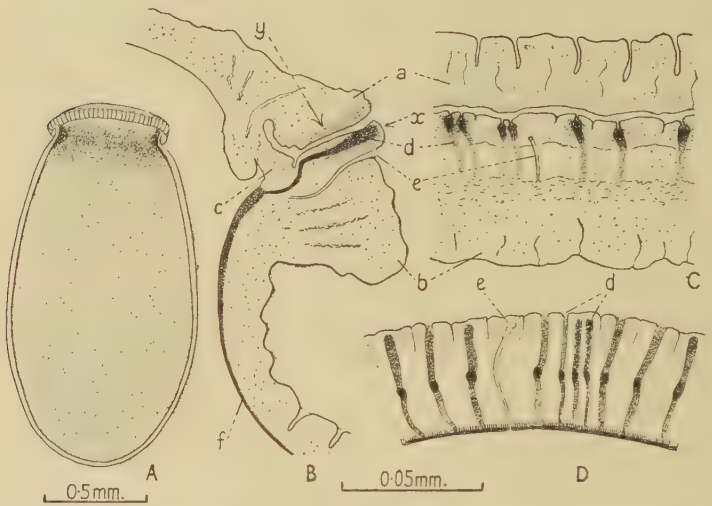
this could be seen to spread backwards inside the shell; whence he concluded that the film of air which he postulated does in fact exist (cf. Leuckart, 1855; see discussion, p. 448).

One of us (Wigglesworth, 1950) has recently described a method for filling the tracheae and tracheoles of insects with cobalt sulphide, which makes



Text-fig. 1. A, egg of *Rhodnius* injected with cobalt sulphide, ventral view; showing the pseudomicropyles traversing the rim, and the injected layer inside the shell. B, longitudinal section of junction between the cap and the rim of the shell. C, optical section of cap and rim of the shell viewed from the direction *x* in B. D, optical section of the rim viewed from the direction *y* in B. *a*, cap; *b*, rim of shell; *c*, sealing bar between cap and rim; *d*, pseudomicropyles or Leuckart's canals; *e*, true micropyle; *f*, spongy inner layer (resistant protein layer) filled with the cobalt sulphide.

possible the demonstration of thin films of air in the form of permanent preparations. It is the object of this paper to describe the distribution of air in the egg of *Rhodnius* in relation to the structure of the shell as worked out in detail by Beament (1946*a*, 1947) and to describe comparable respiratory mechanisms in a number of other insect eggs.

Respiratory mechanism in the egg of Rhodnius prolixus

Recently laid eggs of *Rhodnius* were injected with cobalt naphthenate mixed with an equal volume of light petroleum (white spirit or kerosene) (Wigglesworth, 1950), shaken vigorously in kerosene saturated with hydrogen sulphide, gassed with hydrogen sulphide for half an hour, and then fixed in Carnoy's fluid.

Text-fig. 1A shows the anterior region of the *Rhodnius* egg seen from its more elongated ventral aspect. The 'pseudomicropyles' (Beament, 1947), blackened with cobalt sulphide, run inwards from the rim to end in a diffusely impregnated sheet which lines the shell. This inner sheet is evidently much

thicker beneath the neck of the egg, where it appears as a dark-grey collar; it becomes paler posteriorly, but it extends as a thin grey layer around the entire shell. As seen in surface view this grey layer shows a finely punctate appearance. The points are larger along the boundaries corresponding with the follicular cells so that these show up as a grey network. This appearance is very evident around the neck of the egg; it is not detectable farther back.

The relations of the parts are best seen in longitudinal sections of the shell (Text-fig. 1B). Where the margin of the cap and the margin of the rim adjoin, the substance of the shell is diffusely impregnated. The outer part of the pseudomicropyle is hardly visible in this diffusely impregnated zone. But in the inner part, particularly where it curves round the 'sealing bar' between the rim and the cap, it is very conspicuous. It becomes narrower at the inner end where it joins the impregnated inner sheet. It can now be seen that this sheet, thickened and ending abruptly just behind the sealing bar, and becoming thin posteriorly, is the layer described by Beament (1946*a*) as the 'resistant protein layer'. In this same section a true micropyle with its funnel-shaped opening can be seen a little behind the pseudomicropyle. Its contents are a very pale grey, showing that it is filled with a substance that is only slightly impregnated with the sulphide.

Text-fig. 1, C and D, shows the pseudomicropyles from two other aspects. In Text-fig. 1C the margin of the cap and the rim of the shell are seen from the direction *x* in Text-fig. 1B. The pseudomicropyles appear as deeply blackened canals running through the impregnated substance of the shell and then curving backwards. Toward their outer extremities the pseudomicropyles are cleft in their long axis so that the lumen communicates with the space between the rim and the cap.

In Text-fig. 1D the rim of the shell is seen from the direction *y* in Text-fig. 1B. Some of the pseudomicropyles are open or cleft at the margin of the rim, others have rounded closed extremities. But all lie in deeply impregnated shell substance which extends to the surface. At their inner extremity they end in the substance of the resistant protein layer. To reach this layer they pass through an impregnated layer with vertical striations. Perhaps this represents the presence of air in the pore canals of the innermost part of the lipoprotein layers, which come very close to the inside of the shell in this region of the egg (Beament, 1947). A funnel-shaped true micropyle can be seen beyond the pseudomicropyles.

It is evident that in the *Rhodnius* egg there is no free layer of air. The air, and the cobalt sulphide which replaces it, merely permeate the substance of a solid protein layer. Even the pseudomicropyles have not the jet-black appearance they would have if they contained only air. The impregnated substance may be conveniently referred to as 'spongy' or 'porous' protein. The intensity of blackening which it shows after impregnation with the cobalt sulphide is a measure of its sponginess or porosity. The main layers of the exo- and endochorion are glassy in appearance and admit no sulphide. The cement which fills the pits on the shell and occludes the true micropyles shows

only the faintest shade of grey, indicating an almost negligible porosity. The tanned 'resistant protein layer' is highly porous; in optical section it appears a uniform dark grey, but in surface view it shows a finely granular appearance.

Eggs removed from the calyx before laying, that is, eggs fully formed and waterproofed but not yet coated with cement, were dried in air for 24 hours and then injected with cobalt sulphide. In these the sulphide filled the pits in the shell; the impregnation of the pseudomicropyles, &c., occurred as in the egg after laying; the true micropyles were rather more deeply impregnated—but even at this stage they were clearly filled with some solid material and not with air alone.

These observations confirm in principle the conclusions of Tuft. Outside the waterproofing layer of wax supported by the epembryonic membrane (Beament, 1949) there is a continuous layer of air-containing porous protein communicating with the atmosphere by way of the pseudomicropyles, which are likewise filled with dry porous material.

Cimex lectularius

The egg of the bed-bug was described and figured by Leuckart (1855). There are something like 100 canals around the rim of the shell, resembling those in *Reduvius* so closely that Leuckart concluded that the Cimicidae and the Reduviidae must be nearly related.

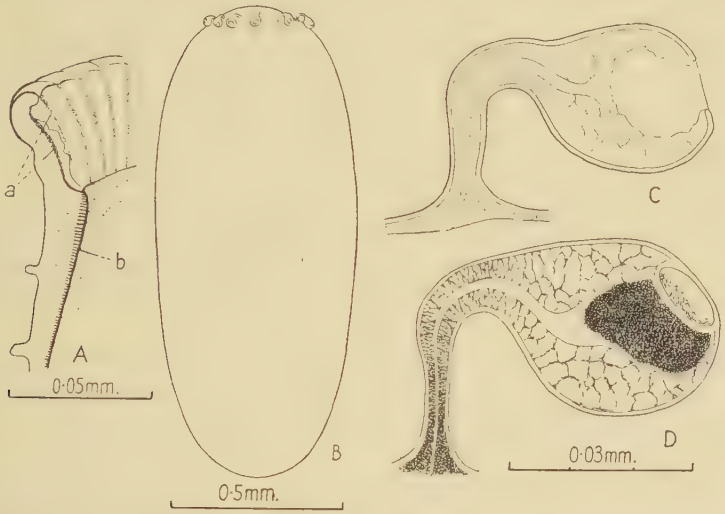
After injection with cobalt sulphide (Text-fig. 2A) the ring of grey, faintly granular pseudomicropyles can be seen to run from the inner surface of the margin of the rim of the shell. They are slightly club-shaped near the outer extremity, but taper inwards to end just behind the neck of the egg in a dark granular layer. In optical section this granular zone is seen to be made up of dark rods in a pale matrix, with a very thin continuous dark inner border. This layer becomes gradually thinner behind the neck and the granular rodded appearance vanishes, but a darkly injected inner layer surrounds the entire egg.

The pseudomicropyles or Leuckart's canals, like those of *Rhodnius*, fail to pierce the innermost layer of the chorion. They cannot therefore serve as micropyles. No true micropyles have been observed. The egg of *Cimex* is fertilized in the ovary (Cragg, 1920, 1923; Abraham, 1934); perhaps no micropyles are present.

Oncopeltus fasciatus

The egg of this Lygaeid, the large milkweed bug, was figured by Heidemann (1911). It has a ring of a dozen pipe-shaped processes just behind the margin of the cap (Text-fig. 2B). These are the 'sperm cups' (Samenbecher) of Leuckart (1855), characteristic of many Hemiptera. Each cup (Text-fig. 2C) has a thin refractile wall continuous with that of the shell. Inside this is a coarse reticulum surrounding a clear zone which runs like a funnel down the stem and through the shell. The reticular meshwork of the wall becomes progressively closer as it extends along the stem to fuse with the inner layer of the shell. A round opening forms the mouth of the cup.

After injection with cobalt sulphide (Text-fig. 2D) the vestibule of the cup filled with a dense black plug; but the duct remains colourless: clearly it is mpletely blocked with some impermeable substance. On the other hand, the eshwork of the walls is freely impregnated with the sulphide. The walls ecome progressively darker as they approach the base, and where the stem ins the shell they merge with a fine impregnated reticulum which forms the



EXT-FIG. 2. A, rim of the shell in egg of *Cimex* after injection with cobalt sulphide, seen in tical section with part of the rim beyond. *a*, Leuckart's canals; *b*, injected spongy layer with ected rods running into the shell. B, egg of *Oncopeltus* showing the 'sperm cups' at the terior pole. C, optical section of sperm cup showing central micropylar canal and thick ongy walls continuous with the inner part of the shell. D, the same after injection with balt sulphide; the spongy substance of the wall is filled with the sulphide, and there is a solid plug of sulphide in the vestibule but none in the micropylar canal.

ner layer of the chorion. In the anterior part of the egg the impressions of e follicular cells can be seen as darker outlines in this reticulum. Behind is zone the meshwork becomes gradually finer and over the greater part of e egg it appears, even under the oil immersion, as a homogeneous grey layer hich extends all round the inside of the chorion. The total thickness of the orion is about 3μ of which the porous impregnated layer makes up about ie-sixth, say $0{\cdot}5\mu$.

Eggs were removed from the calyx of the *Oncopeltus* female. In them the ps have much the same appearance as in the egg after laying, but the lumen funnel-shaped and extends without interruption from the opening of the p, down the stalk and through the shell. The walls of the cup and stem have e same sponge-like appearance as in the egg after laying. On drying in the the entire contents and the spongy walls of the cup shrink away and leave ly a collapsed dry residue.

These eggs from the calyx, when dried in air, resist shrinkage for some

hours. Like the corresponding eggs in *Rhodnius* they are already waterproofed (Beament, 1946*b*). But waterproofing is incomplete, for after 24 hours they have collapsed—perhaps they dry up through the open cups. When these collapsed eggs are injected with cobalt sulphide the entire cup contents and stem contents are deeply blackened throughout.

At the time of laying the egg is coated with cement; from the foregoing observations it is evident that this not only covers the shell but runs into the micropylar cups and, as the egg dries, comes to fill the lumen of the duct. Hence, when the egg is injected with cobalt sulphide after laying, this forms a solid plug in the vestibule of the cup, but there is no injection of the glassy substance filling the lumen, or of the spaces in the spongy walls. The diffuse grey impregnation is confined to the porous protein forming the meshes of the sponge.

Apparently the cups serve both as micropyles and as respiratory organs. The funnel-shaped lumen forms the micropylar duct. This duct is occluded with cement after laying, and it is the porous substance of the wall of the cup and its stem which conducts air into the porous lining of the shell.

Psylla mali

The egg of the apple sucker has been described by Speyer (1929). In surface view the chorion shows irregular areas of about 7–10μ in diameter surrounded by refractile walls. On focusing down, these areas are seen to enclose smaller areas and, focusing down again, each of these contains a group of small pale channels.

After injection with cobalt sulphide with the minimum of washing, it is not possible to detect any obvious impregnated layer lining the chorion. But the refractile constituent of the shell is everywhere impregnated. The chorion is about 3–4μ thick. It is made up of a continuous porous substance, readily impregnated with cobalt sulphide, which encloses little vertical pillars of glassy unimpregnated material. No one part of the egg appears to be specialized for respiration; but the bulk of the substance of the shell is porous and conveys air to the interior.

Locusta migratoria

Eggs of the migratory locust were removed from the egg pods at times varying from 1 to 14 days after laying, and injected with cobalt sulphide. This extends everywhere beneath the dry and shrivelled chorion of the older eggs but there seem to be no air channels passing through the substance of the serosal cuticle which forms the chief protective membrane of the grasshopper egg (Slifer, 1937).

Dixippus morosus

Structure of egg-shell. The egg coverings of *Dixippus* were described by Elkind (1915) and her results confirmed by Cappe de Baillon (1927) and Leuzinger (1926). The hard shell or 'exochorion' is composed of three layers:

an outer layer, amber coloured, vertically striated, with a rugose surface; a middle layer, thick and fibrous and impregnated with lime; and an internal layer, very thin and translucent. Beneath the shell is a thin, soft 'endochorion' consisting of two delicate membranes which are readily detached from the exochorion and separated from one another. The outer membrane was described as 'homogeneous and transparent', the inner as 'opaque and porous'. (In Leuzinger's (1926) description these layers appear reversed.) Beneath the rim of the cap the endochorion has an annular thickening; below the cap itself it is not differentiated into two layers but is clear and homogeneous throughout.

These descriptions have been confirmed and extended. The outer rodded amber layer of the shell is about 10μ thick; it is very tough and is apparently composed of lipoprotein from which oily droplets are liberated on warming in nitric acid saturated with potassium chlorate. The middle layer is about 40μ thick; it is composed of rather soft protein associated with lime and contains numerous air-filled cracks. It dissolves rapidly in 10 per cent. sodium hypochlorite to which 30 per cent. sulphuric acid is added drop by drop. The inner layer is about 10μ thick; it consists of hardened (probably tanned) protein and is slowly soluble in concentrated nitric acid, but contains no detectable quantities of oil.

In the elongated area or 'scar' around what is commonly regarded as the micropyle (Leuckart, 1855) the outer and inner layers of the shell appear to be missing. The shell has a chalky white appearance due to the extensive air-filled cracks in the middle layer; and on immersion in the hypochlorite-sulphuric acid mixture the micropylar plate drops away from the shell. (The mealy white character of the inner parts of the scar was noted by Johannes Müller (1825) in other Phasmids; the white substance was stated by Leuzinger (1926) to be 'soluble in xylol'—a result, in fact, of the displacement of air by the oil.)

Where the margin of the cap joins the rim of the shell there is a ring of broad silvery white lines traversing the shell and leading to the thickened substance of the rim which likewise is silvery white. The silvery appearance of these structures is due to an exaggeration of the air-filled cracks in the shell. At this point the cracks appear to extend through the outer layer to make contact with the atmosphere. The knob on the cap of the egg (fully described by Leuzinger (1926)) is soft and sponge-like. It contains air in its meshes which can be squeezed out.

The space between the shell and the 'endochorion' is occupied by a thin layer of grease. The endochorion floats away from the shell at once if this is immersed in chloroform or other wax solvent; and if the membrane is stripped from the shell while this is held at the surface of water, a film of grease can be seen to float up from the intervening space. This layer of grease appears to waterproof the egg at a very early stage in the formation of the shell. Eggs removed from the follicles at all stages in the deposition of the shell can withstand immersion in distilled water for 24 hours without rupture, even at a stage when the shell is still soft.

As will be described more fully below, in the region of the scar the two layers of the endochorion are firmly bound together and at the 'micropyle' itself a short cord connects the endochorion to the shell. The glassy outer wall of this cord is continuous with the outer layer of the endochorion; the opaque, white, air-containing medulla becomes continuous with the inner layer.

Distribution of air in the egg. If the shell is dissected away under water, the endochorion can be seen as a silvery membrane, obviously containing air. The silvery appearance ends abruptly in a conspicuous white ring below the rim of the cap. The glassy membrane below the cap itself clearly contains no air. No air bubbles come away from the surface of this membrane; but if it is ruptured with needles, a little air may be set free from between its component layers.

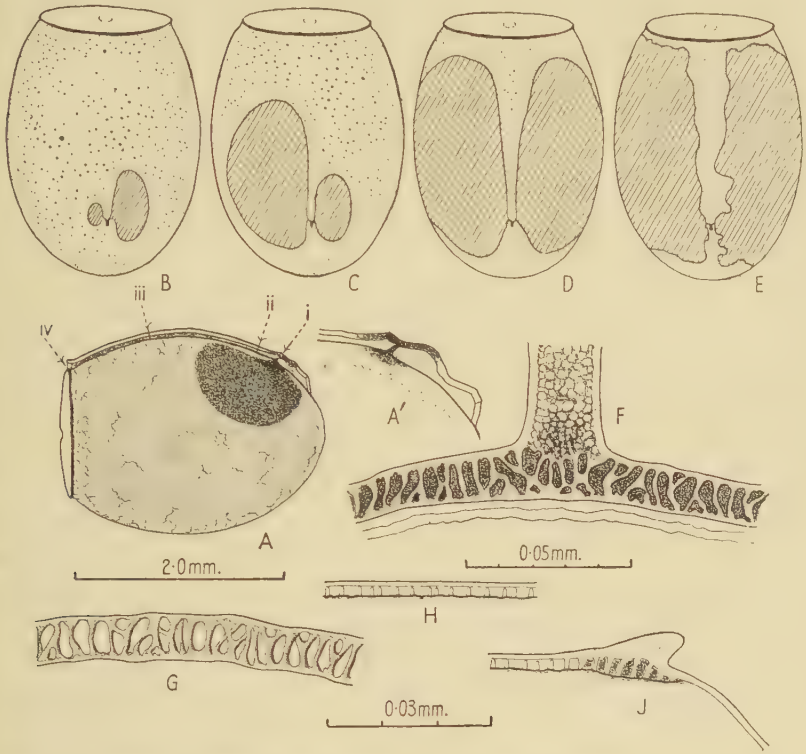
The endochorion is clearly a flattened sac containing air in its substance and a little air between its walls. This sac is connected to the exterior at the so-called 'micropyle', which, as shown by Hahn (1922), forms the respiratory opening.

The distribution of air was further studied in eggs injected with cobalt sulphide. The silvery ring and the radiating lines in the rim and the margin of the cap were dark grey: the air in the cracks of the shell in this region had been filled; but the injection did not extend very far back from the rim and there was no layer of sulphide in or below the inner layer of the shell. The knob of the cap (which was regarded by Cappe de Baillon (1927) as a respiratory structure) became impregnated with sulphide in some eggs, not in others. In no case was there any film of sulphide below the cap or connected with the knob.

On the other hand, the pore of the 'micropyle' is always deeply impregnated with sulphide, and where the duct from the pore joins the endochorion its dark contents spread out over the two sides of the egg to form oval black patches of varying extent (Text-fig. 3A). Beyond the limits of these patches the inner membrane of the endochorion is coloured a more or less uniform grey which extends as far as the annular rim which is a deeper grey. The disk below the cap is colourless. These results confirm the observations on the freshly dissected egg and prove that the endochorion forms an air-containing sheath around the embryo, connected with the atmosphere at the 'micropyle'.

Text-fig. 3, B–E shows the endochorion in four eggs, injected with cobalt sulphide, with the embryo at four different stages of development. It can be seen that the black 'pools' on either side of the micropyle become progressively larger as the embryo grows, until they extend over the greater part of the endochorion. As development proceeds, and the egg contents diminish, the space created is occupied by free air which collects between the separated layers of the endochorion—just as air collects in the egg of the bird. By transillumination in a strong light it is possible to see these expanding air spaces in the intact egg. Their margins are always smooth and sharply defined, showing that beyond their limits the space between the two layers of the endochorion must be occupied by liquid.

Structure of the endochorion. It is clear that the endochorion forms a pneumatic sheath for the embryo. The structure of this sheath and the distribution of air within it have been studied in sections after injection with cobalt



Text-fig. 3. A, egg of *Dixippus* after injection with cobalt sulphide; the shell (exochorion) has been removed except along the upper margin and only the endochorion remains. Note the sulphide-filled pool in the region of the respiratory pore, the uniform injection of the endochorion elsewhere, the deeply injected ring below the margin of the cap, and the absence of injection below the cap itself. A′ shows the detail of the attachment of the endocuticle to the shell at the respiratory pore. B, C, D, E show the endochorion viewed from above in eggs injected at four stages of development: B is a newly laid egg; E is an egg very near to hatching. The shaded areas show the extent of the sulphide-filled pools on either side of the respiratory pore. F, transverse section of the injected endochorion in the mid-dorsal line at the position i in A, showing the respiratory pore, the branching columns uniting the inner and outer walls of the endochorion and the sulphide deposits between. G, section of the same at position ii in A, showing branching columns with injected walls holding together the layers of the endochorion. H, ditto at position iii in A, showing clear outer layer, inner layer impregnated with sulphide and bearing conical projections. J, ditto at position iv in A; outer layer thickened to form ridge which is bound to inner layer by columns with deposits of sulphide between; below the cap (to the right) the impregnated inner layer is absent.

sulphide. The respiratory pore consists of a densely impregnated reticulum enclosed in the colourless glassy outer membrane (Text-fig. 3F). Below the pore and in the surrounding zone, particularly in the mid-line anterior to the pore, the inner and outer membranes of the endochorion are bound together

by branching columns, the walls of which are impregnated with sulphide (Text-fig. 3G). At some little distance from the micropyle these columns gradually become detached from the outer membrane and their covering is no longer impregnated. Finally they are replaced by small conical elevations of refractile yellowish material, their apices in contact with the outer membrane but not united with it, their bases inserted upon the very thin deeply impregnated inner membrane (Text-fig. 3H).

The total thickness of the combined layers of the endochorion is about $2{\cdot}5\mu$. The conical elevations vary from 1 to $1{\cdot}5\mu$ in diameter at the base; they are about 2μ in height. The impregnated inner membrane appears to be not more than $0{\cdot}1\mu$ in thickness. In the region of the lateral 'pools' of air, the two layers are separated and there are irregular deposits of cobalt sulphide among the conical knobs.

Around the thickened rim, where the two layers are once more bound together by vertical columns, there are more deposits of cobalt sulphide between the layers—confirming the presence of free air within the rim (Text-fig. 3J). Beyond this point the inner layer is absent; the disk below the cap is made up only of the glassy outer layer which contains no sulphide.

The structure of the endochorion has been further studied with the electron microscope. The outer membrane shows a faintly vacuolated appearance but no other structure (Pl. I, fig. 1). In the inner membrane (Pl. I, fig. 2), the conical elevations show up as opaque disks and in the membrane on which they rest there are small vacuolar spaces. Pl. I, fig. 4 shows an unfixed preparation of the inner membrane from an egg injected with a solution of lead naphthenate containing 12 per cent. of metal, converted to the sulphide. The elevations appear as before; the vacuolated spaces in the intervening membrane are now filled with the opaque lead and, in addition, there is a finer lead-filled vacuolation consisting of spaces perhaps one-hundredth of a micron in diameter. Pl. I, fig. 3 shows a preparation similar to Pl. I, fig. 2 after shadowing with gold and palladium at an angle of 45°. The conical shape and variable height of the projections is well shown; they are seen to be about twice as high as they are broad at the base. Some very minute conical projections are also present.

These preparations must be interpreted with caution, but it would appear that the porous inner membrane contains a labyrinth of air-filled spaces (filled with lead in Pl. I, fig. 4) ranging from one-hundredth to one-tenth of a micron in cross-section.

Chemistry of the endochorion. The outer membrane of the endochorion appears to consist of a protein of 'keratin' type, resembling the epembryonic membrane of *Rhodnius* (Beament, 1949); it is very tough and resists solution in mineral acids and in alkali; but it is softened by thioglycollic acid and it then becomes readily soluble in acids and alkalis and readily stainable in acid fuchsin and other dyes. The inner membrane bearing the knobs consists of a soft lipoprotein from which oil is liberated by treatment with 10 per cent. sodium hypochlorite or with concentrated nitric acid alone. Inside the inner

membrane there is an extremely thin layer of protein containing polyphenol, which turns brown in ammoniacal silver nitrate. This extends over the whole inner surface of the egg, including the cap. In eggs containing advanced embryos this innermost layer appears to have a thin covering of wax.

First appearance of air in the egg. The female *Dixippus* has an ovary of primitive type in which the ovarioles open along the side of a linear calyx or oviduct. In a female at the height of egg production, none of the eggs in the follicles, even when they have fully developed and opaque shells, contain air in the endochorion. Of the eggs in the linear calyx some already contain air, others do not, although all are bathed in fluid. It may therefore be concluded that air first appears in the endochorion at some time after ovulation, but well before the egg is laid, and that its first appearance is not connected with the exposure of the egg to the atmosphere.

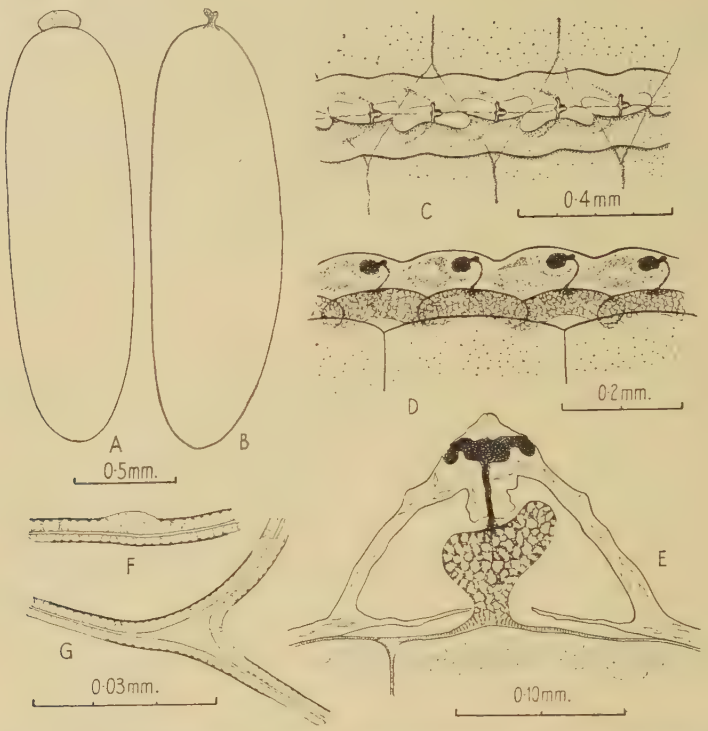
Blattella germanica

Respiratory mechanism. The formation of the oötheca of the German cockroach was well described by Wheeler (1889), but no attention has been given to the respiratory apparatus of the eggs since the incomplete account by Leuckart (1855). Each egg has a curved elongated expansion of the chorion which forms a vacuolated ridge along its upper pole (Text-fig. 4, A and B). This excrescence was regarded by Wheeler (1889) as a mass of degenerated nurse-cells; but, as can be seen by observing the eggs in the ovary, it is a true part of the chorion, laid down by a special group of follicular cells. At the point of its attachment, and on either side, the chorion is somewhat thickened; but over the greater part of the egg the chorion is exceedingly thin. These vacuolated expansions lie below the crista of the oötheca. In the fresh state, as noted by Leuckart (1855), they have a silvery-white appearance, evidently due to their substance containing air.

If the crista of the living oötheca is examined in water, a tiny air-filled cavity can be seen above each egg, overlying the vacuolated ridge on the chorion (Text-fig. 4C). This cavity has a characteristic shape, consisting of an oval median space and two little antero-lateral expansions with rounded ends. In transverse sections through the crista it can be seen that these lateral expansions penetrate so deeply into the substance of the oötheca that the wall of the chamber is incomplete over their upper and outer extremities (Text-fig. 4E). At this point they actually pierce the side wall of the crista. Clearly these chambers form the respiratory openings of the oötheca and convey air to the vacuolated structures below and so to the membranes around the egg.

These conclusions are readily confirmed in oöthecae injected with cobalt sulphide. The little chambers are filled with the black sulphide; and when examined in side view a thin curved 'duct' can be seen leading from the posterior extremity of the median cavity to the mid-point of the vacuolated ridge below (Text-fig. 4D). There is often a dense black area around the point of entry of the 'duct' into the ridge. The rest of the vacuolated excrescence is heavily impregnated with the sulphide which fills the meshwork of the sponge,

leaving clear spaces between (Text-fig. 4E). The dilated chorion running outwards from the point of attachment of the ridge is similarly impregnated. The rest of the chorion shows a grey impregnation as described below.



TEXT-FIG. 4. A, dorsal view of egg of *Blattella* showing vacuolated excrescence at the anterior pole. B, lateral view of the same. C, a portion of the crista of the living oötheca seen from above. The terminal excrescence on each egg appears as a curved white object, above which is a little T-shaped air chamber. D, side-view of part of crista after injection with cobalt sulphide; a curved duct leads from the sulphide-filled air chamber above to the vacuolated excrescence which is likewise heavily impregnated with sulphide. E, thick transverse section (75 μ) through crista of injected oötheca. The sulphide-filled chamber above opens to the exterior through lateral pores; its duct leads down to the vacuolated excrescence which appears pear-shaped in cross-section and becomes continuous with the thin chorion. F, section through the chorion of two adjacent eggs after cobalt sulphide injection. Grey impregnated columns traverse the chorion; the larger columns above mark the boundary between follicular cells (cf. Pl. I, fig. 6). A deeply injected film lies inside the chorion and this film is thickened at the end of each spongy column. G, ditto showing three adjacent eggs with cement between them.

The fresh oötheca examined in water has a silvery sheen due to the presence of air in the egg membranes. If the capsule is dissected in saline the distribution of this silvery film of air can be readily seen. It varies considerably from one part of the chorion to another. In general, the film is less complete in recently laid eggs than in those at an advanced stage of development. In some places it has a regular net-like arrangement, being confined to the boundaries of the impressions left by the follicular cells (Pl. I, fig. 5). In other

places it is continuous over the cell areas, and the boundaries for the most part appear dark. But most commonly it is quite irregular in its distribution: areas with a complete film link up by means of irregular connexions with areas showing a confused network (Pl. I, fig. 6).

Wheeler (1889) believed that the chorion of *Blattella* consists of two thin laminae kept in apposition by minute trabeculae or pillars; the trabeculae being larger and more widely spaced along the boundaries between the cell areas. When dried fragments of the chorion were immersed in glycerol, he believed that he could see this creeping between the trabeculae and displacing air from between the laminae.

This experiment has been repeated, and although the appearances could readily be interpreted as was done by Wheeler, it is impossible to determine with certainty the true relations between the columns and the air in such preparations. When the adherent choria of adjacent eggs are examined in glycerol it can be seen that the two air layers are separated by an appreciable distance (cf. Pl. I, figs. 5 and 6). By focusing on a fine adjustment with a measuring scale it was estimated that there is an interval between them of about 3–4μ. This agrees well with the thickness of the two choria combined and is evidence that the air is confined to the innermost layer. If it occupied the spaces between the columns of the chorion, the two air films would be separated by less than 1μ.

If dry pieces of chorion are immersed in the cobalt naphthenate in white spirit this displaces the air very rapidly, usually covering the cell areas first and later the boundaries. If the fragments are then quickly rinsed in a heavier petroleum oil and gassed with hydrogen sulphide the above conclusions are confirmed. In surface view the trabeculae are seen as dark disks against a uniform grey ground. In optical section they appear as grey columns in a glassy matrix with a continuous black impregnated film over the surface.

Oöthecae, injected with cobalt sulphide while intact, have been cut into horizontal sections and the same results obtained. The chorion is 1·5–2μ thick. It is traversed by grey impregnated columns, dilated at their inner and outer extremities, embedded in a colourless substance. The columns are thicker, more prominent and more widely separated along the boundaries left by the follicular cells. At the inner end of the columns the substance appears often to have shrunk and left a tiny pit filled with air which has been replaced by sulphide. In many places the impregnated film is continuous over the surface of the whole area of one or more cells, forming a layer not more than 0·1μ thick. There are similar minute deposits of sulphide at the outer ends of some of the columns; but there is never a continuous film on this outer surface of the chorion. Adjacent eggs are firmly bound together by an amber-coloured material. Normally this substance forms a very thin layer (say 0·5μ thick), but where three eggs meet it may be quite conspicuous (Text-fig. 4G).

There can be no doubt, from these observations, that the chorion is a solid structure consisting of columns of air-containing spongy protein, embedded

in a glassy matrix, and with a thin layer of spongy protein, more or less extensively filled with air, over the inner surface.

Chemistry of the oötheca and chorion. As was shown by Pryor (1940) the oötheca of the cockroach consists of 'sclerotin' (protein tanned by quinone) containing, embedded in its substance, the familiar crystals of calcium oxalate. The inner parts of the oötheca, which contain no crystals, and the amber substance which binds the eggs together, are readily dissolved in 5 per cent. potash acting at room temperature for 2 or 3 minutes. By this means the eggs can be separated from one another and from the oötheca: for the outer parts of the oötheca resist solution in cold 5 per cent. potash for some hours.

If the chorion is isolated and is then exposed to dilute sodium hypochlorite, the outer part is first dissolved (without liberation of oil) and a structureless inner membrane remains. This is the innermost layer which contains air; it appears to have the same chemical properties as the pneumatic inner layer of the endochorion in *Dixippus* (p. 438).

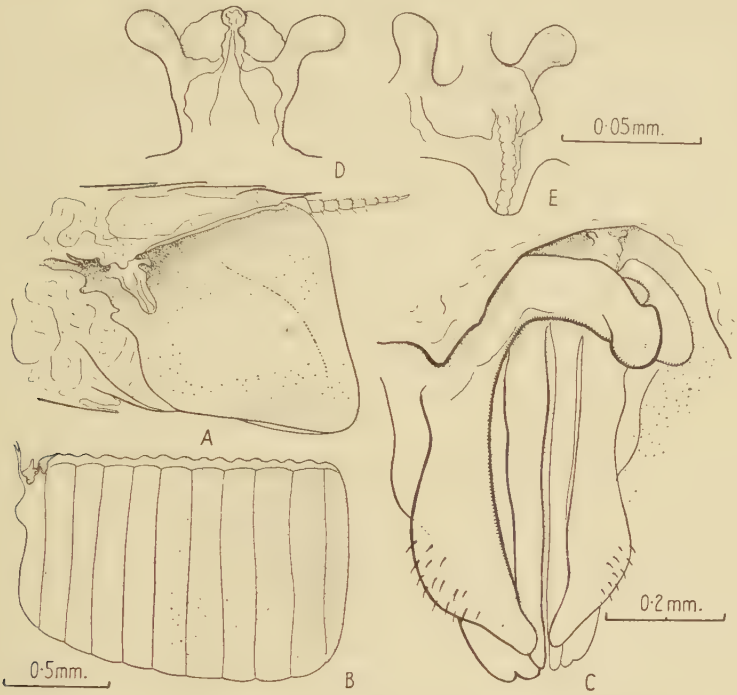
The vacuolated ridge on the upper pole of the egg is the most resistant structure of all. It withstands hot concentrated potash longer than the outer parts of the oötheca, and it is more resistant to mineral acids and to strong oxidizing agents than any of the other layers. It will dissolve, however, on warming in concentrated nitric acid containing potassium chlorate, and oil is set free in the process. It is composed presumably of lipoprotein. The fact that it will swell somewhat in distilled water suggests that it may consist of untanned lipoprotein.

Formation of the respiratory apparatus. The formation of the oötheca in *Blattella* was admirably described by Wheeler (1889) and brief accounts are given by Kadyi (1879) and Chopard (1938); but it was of interest to discover the mode of formation of the remarkable respiratory structures described above. The identical form of the respiratory apparatus above each egg in a given oötheca suggests that it is moulded upon a single die, and this has proved to be the case.

In the female cockroach examined during the height of oviposition, the genital chamber is an oval cavity holding the half-formed oötheca (Text-fig. 5A). From the roof of this chamber at its anterior extremity the elongated finger-like genital appendages project downwards into the soft portion of the oötheca in process of formation, and hold the latest egg in position. Dorsally, towards the base of these finger-like appendages is a pair of thumb-like projections directed backwards (Text-fig. 5C). It is these which serve to mould the upper cavity of the oötheca; they hold between them the vacuolated expansions at the upper pole of the chorion (indeed, their inner aspect is specially shaped for this purpose) and they thus serve to orientate the egg in the oötheca.

At the root of these thumb-like lobes and immediately below the narrow roof of the genital chamber is a very small median lobe with a tiny sclerotized horn projecting on either side (Text-fig. 5C). This structure has the exact form of the respiratory chambers and is clearly the die on which they are

moulded. Below and behind this median lobe is a thin membrane with a thickened rounded posterior margin (Text-fig. 5, D and E). It is this rounded margin which serves as the die for the curved 'duct' that leads the air from the respiratory chamber to the expansion of the chorion.



TEXT-FIG. 5. A, longitudinal section of genital chamber of female *Blattella* showing the group of genital appendages projecting downwards at the anterior end. B, half-formed oötheca removed from the genital chamber shown in A. The leading (posterior) extremity is becoming hardened; at the soft anterior extremity can be seen the orifice from which the genital appendages have been withdrawn. C, oblique posterior view of the genital appendages with the pair of curved thumb-like processes directed backwards at their base, and above these the little horned die for moulding the respiratory chamber. D, ventral view of the horned die. E, oblique posterior view of the same showing, below and behind, the thickened rounded strand which moulds the duct.

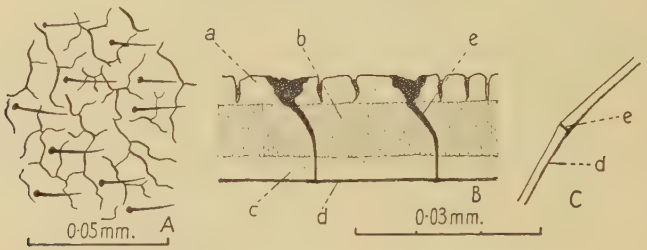
The accessory glands which pour out the substance of the oötheca discharge at the base of the genital appendages. It can now be seen how this secretion will be moulded by the little horned die to provide the respiratory chamber and respiratory duct for the egg below. Moreover, the upper and outer extremities of the horns will press against the roof of the genital chamber and will thus give rise, when they are withdrawn, to the two minute apertures by which air can enter the oötheca.

Immediately above the roof of the genital chamber in this region are some more glands which discharge their secretion at the base of the middle lobe with the horns. Perhaps this secretion serves as a cement which sticks the two

lips of the oötheca together. The source of the cement between the eggs, which is so much less resistant to solution than the outer parts of the oötheca, has not been investigated.

Adalia bipunctata

The only coleopterous eggs examined were those of the two-spot ladybird. When newly laid these show no air in the shell. After development has proceeded for a day or two and the serosal cuticle has been laid down, the micropylar tubes forming a circlet at the anterior pole are filled with air and this can be seen to extend over the surface of the egg between the very thin chorion



Text-fig. 6. A, chorion of *Bombyx* after injection with cobalt sulphide seen from the inside. The irregular clefts in the shell and the tapering respiratory canals are injected. B, transverse section of the same. C, transverse section of chorion of *Ephestia* egg after cobalt sulphide injection. *a*, outer layer of chorion with clefts; *b*, middle layer with pore canals; *c*, inner layer; *d*, innermost spongy layer impregnated with sulphide; *e*, respiratory canals filled with impregnated porous substance.

and the serosal cuticle. When the eggs are injected with cobalt sulphide this irregular layer of air between chorion and serosal cuticle is filled, but no 'porous' layer is present in either membrane.

Bombyx mori

Leuckart (1855) examined the structure of the chorion in a great many lepidopterous eggs, including that of the silkworm; and in nearly all of them he found numerous tapering canals, scattered over the surface of the egg, conveying air to the inside of the chorion. In some eggs he noted the true 'pore-canals' which also appeared to contain air in some species, so that the whole substance of the shell was aerated. The respiratory pores in the silkworm egg were again described by Verson (1893).

The shell of the silkworm egg is about 18μ thick (Text-fig. 6B). It consists of three main layers: (*a*) an outer layer of about 4–4·5μ, homogeneous and refractile with irregular clefts extending down to its inner limit; (*b*) a middle layer of about 10μ with conspicuous pore canals ending abruptly at the inner limit of this layer; and (*c*) an inner homogeneous layer of 3–4μ. The shell is pierced by some hundreds of respiratory canals, distributed all over the surface of the egg, with the exception of the micropylar region, and not confined to the lenticular surfaces as claimed by Verson (1893).

When injected with cobalt sulphide the funnel-shaped respiratory canals are very conspicuous (Text-fig. 6A). They appear not to contain air alone but a porous protein filled with air. They lead from the exterior to the inner part of the deepest layer of the shell; and the innermost part, extending all round the shell to a depth of about $0{\cdot}5\mu$, is likewise impregnated with the sulphide—often most deeply around the point of entry of the respiratory filaments (Text-fig. 6B). Clearly these porous filaments conduct air to a pneumatic membrane that lines the shell.

The clefts in the outer layer of the shell retain a variable amount of sulphide and so do the pore canals. The silvery appearance of the shell when examined in water in the fresh state results from the presence of air in all these parts; but the true respiratory structures are the tapering filaments and the porous inner membrane to which they lead.

Ephestia kuehniella

The egg of *Ephestia* was described and illustrated by Lehmensick and Liebers (1937) and by Müller (1938). The chorion is thrown into star-shaped folds, about sixty in number, and at the central point of each star there is a minute pore. When injected with cobalt sulphide the pores appear as deeply impregnated canals which lead through a colourless chorion of about 3μ in thickness to a very thin impregnated layer that lines the shell (Text-fig. 6C). This layer is thickened below the stellate folds so that the injected egg shows a number of grey stars over its surface.

If the living egg is examined in water by transmitted light the air in the inner layer of the shell gives it a dark appearance in the form of a stellate network with anastomosing rays. This dark appearance is no longer seen after the air has been displaced by immersing the egg in kerosene.

Syrphus sp.

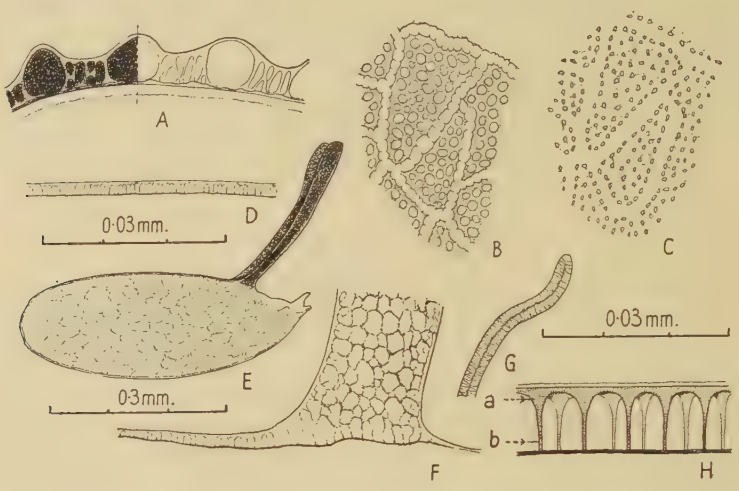
Pantel (1913) described the chorion in parasitic Diptera as made up of two thin laminae, the outer of which is the more robust, bound together by vertical pillars. The space between the pillars at first contains liquid; subsequently the shell is 'pneumatisé' and the liquid is replaced by air.

This state of affairs has been found to exist in the egg of an aphidiphagous Syrphid closely resembling that of *Syrphus luniger* (Bhatia, 1939). Text-fig. 7A shows a transverse section through the shell. The elevated regions corresponding with the boundaries of the follicular cells are hollowed out and filled with air. From the floor of the depressions in the shell, irregularly branching and anastomosing columns run inwards to connect the outer and inner laminae of the chorion. The space between the columns is likewise filled with air.

If minute drops of kerosene are applied to the surface of the egg they do not enter the air-filled cavities. But if the intact egg, together with the leaf on which it rests, is immersed in kerosene, this enters the vacuolated chorion and spreads throughout the shell, gradually displacing the air. There can be no

doubt, therefore, that this pneumatic system communicates with the atmosphere. The points of entry are uncertain; they appear to lie chiefly at the micropylar pole.

If the egg is injected with cobalt sulphide these results are confirmed. Dense black deposits fill the cavities around the cell areas and the spaces between the vertical columns (Text-fig. 7A). The surface of the columns and



TEXT-FIG. 7. A, transverse section of chorion and, below, of chorio-vitelline membrane in *Syrphus*. The part to the left shows the appearance after injection with cobalt sulphide which fills the large cavities, the spaces between the columns, the space between the chorion and the chorio-vitelline membrane, and impregnates the surface of the columns. B, surface view of chorion close to micropylar region in *Calliphora* egg after cobalt sulphide injection. The microscope is focused near the surface (cf. *a* in H below). C, the same with microscope focused at deeper level (cf. *b* in H below). D, transverse section through chorion in lateral region of *Calliphora* egg. E, egg of *Drosophila* injected with cobalt sulphide. F, optical section of the same at the base of the horn. G, optical section of the tip of the horn. H, schematic section of chorion in egg of Diptera. It is covered (above) by cement; below this are tapering columns with spongy walls which become continuous with the inner spongy layer. The space between the columns may be filled with liquid or with air. *a* and *b* are the levels of the optical sections shown in B and C above.

the lining of the cavities in general are heavily impregnated with the sulphide. Finally, there is a layer of free air between the chorion and the chorio-vitelline membrane which has been replaced by sulphide.

Calliphora erythrocephala

Eggs of the blowfly injected with cobalt sulphide show a general deep-grey coloration of the chorion except immediately around the micropyle at the anterior pole. The dorsal longitudinal folds show up as dark lines. The inner membrane, the so-called chorio-vitelline membrane, is colourless.

The grey coloration results from a general impregnation of a system of columns in the chorion and of a continuous thin film over its inner surface. Text-fig. 7, B and C shows a surface view of the chorion near the anterior end of

ne of these injected eggs. The cell areas, impressed by the follicular cells, re marked out by their paler boundaries. If the microscope is focused near he surface the appearance seen (Text-fig. 7B) is that of a series of clear spaces nclosed in a grey sulphide-filled matrix. On focusing down the continuous natrix breaks up into a number of isolated columns (Text-fig. 7C).

These results are confirmed in histological sections in the vertical plane Text-fig. 7, D and H). There is a very thin outer layer of spongy sulphide-filled ubstance from which vertical columns run through a glassy material to reach he continuous deeply impregnated inner layer of the chorion. In the outer art of the chorion the grey substance arches over the glassy material; along he cell boundaries the pillars are more widely separated and the arches are twice the normal width. Along the longitudinal folds of the shell the structure is the same but the chorion is thicker and the grey columns are therefore longer.

If droplets of kerosene are applied to the sides of the egg they do not spread or penetrate into the spongy layer. But if they come into contact with the longitudinal folds, the oil can be seen to spread rapidly in all directions beneath the chorion, filling the spongy layer and, presumably, the vertical columns, so that the egg loses its opaque white appearance and becomes clear and translucent. Precisely where the oil enters the shell has not been determined. The whole shell, apparently, serves for respiration; but perhaps the longitudinal folds are specially adapted for this function—as both Leuckart (1855) and Pantel (1913) supposed.

It is evident from these observations that in the egg of *Calliphora* the air is confined to the porous innermost layer of the chorion and to the substance of the vertical columns. But if the chorion is removed and exposed to the air it becomes opaque white, and if it is now filled with sudan black in oil or by the cobalt sulphide technique, all the clear spaces between the columns and along the boundaries of the cell areas are found to be injected. Clearly the 'glassy material' between the columns is mainly water; on drying the detached chorion this is replaced by air. (If eggs in which the chorion has been partially torn away are filled with cobalt sulphide, this will show the normal distribution as described above where the chorion is still applied to the egg; it may fill the cell boundaries and some of the spaces between the pillars where the chorion has been detached and become dry.)

Aeration or 'pneumatization' of the cavities in the chorion does not seem to take place in the normal *Calliphora* egg; but it can be induced experimentally in a rather curious way. If the egg is touched at one pole with a glass rod carrying a minute amount of oil (kerosene containing 20 per cent. oleic acid was used) this will spread throughout the spongy layer and the egg becomes clear. But after leaving in the air for a few minutes, gas appears as a network along the boundaries between the cell areas and gradually spreads inwards among the vertical columns until the whole chorion is filled with air. The explanation of this result is uncertain; but perhaps the permeation of the oil throughout the vertical columns has rendered these hydrophobe and thus

favours the absorption of the fluid by the egg or its evaporation through the outer surface of the chorion.

Drosophila melanogaster

Réaumur (1738) regarded the 'ailerons' or extensions of the longitudinal folds on the eggs of *Scatophaga* as serving to prevent their submergence and asphyxiation—which implies a respiratory function. This view was adopted by Leuckart (1855) and by Pantel (1913). It certainly appears applicable to *Drosophila*. If the egg of this fly, laid on moist filter-paper, is transferred to water, it sinks; but the tips of the silvery air-filled horns remain floating in the surface and clearly serve to convey oxygen to the silvery pneumatic chorion of the submerged egg.

Eggs of *Drosophila* injected with cobalt sulphide show a grey impregnation of the entire chorion with the exception of the thin cap-like area behind the micropyle. The horns are very darkly impregnated (Text-fig. 7E). Under the 2-mm. objective the horns are seen to have a foam-like structure, the sulphide being in the films or meshes of the foam (Text-fig. 7F). This spongy meshwork is very fine against the walls and towards the apex of the horn, much coarser towards the centre and the base. Text-fig. 7G shows an optical section of the flattened extremity of the horn: here it is in the form of a regular palisade of two layers.

At the base of the horn, on its posterior aspect, the chorion gradually thins out and in this region the structure of the shell is readily seen in optical section (Text-fig. 7F). As in *Calliphora* it consists of grey impregnated pillars, tapering inwards, with clear spaces between. A continuous impregnated layer covers the inner surface. In surface view the pillars appear (as in *Calliphora*) as grey refractile points confined to the areas marked out by the follicular cells.

Dissection of the female shows that the chorion does not fill with air while the egg is in the follicle, but the air spreads gradually backwards from the region of the horns while the egg is still bathed in fluid in the calyx. Leydig (1867) and Pantel (1913) found that the eggs of parasitic Diptera likewise fill with air while in the oviducts.

Discussion

It was recognized by Leuckart (1855) in his classic paper on the eggs of insects that the chorion must combine protection for the yolk with provision for respiration and for the entry of the sperm. The most striking respiratory adaptation which he described was that in the eggs of *Nepa* and *Ranatra* where the porous air-filled, inner layers of the shell are connected with the porous medulla of the filaments at the anterior pole of the egg. In the distal half of these filaments the air-containing substance comes into immediate contact with the environment (Leuckart, 1855, Korschelt, 1887). Gross (1900) and Heymons (1906) ascribed a similar respiratory function to the 'Samenbecher' on the eggs of Pentatomidae. Hase (1917) regarded the Leuckart's canals in the eggs of *Cimex* as respiratory; and Kullenberg (1944)

adopts the same view in the case of *Notostira* and other Capsids. The 'pneumatic' nature of the chorion was emphasized by Leuckart (1855) also in the eggs of Diptera, Lepidoptera, and Orthoptera. Pantel (1913) stressed the importance of this function in Diptera, and Cappe de Baillon (1920) described in more detail than Leuckart the air-filled cavities in the outer part of the shell in Tettigoniids.

It has proved difficult, however, in the past to trace the actual distribution of air within the egg. Layers filled with gross amounts of air are so refractile that their limits are hard to define; materials containing air in very finely divided form have a high refractive index which might equally well be due to other causes. In the present work, by the use of the cobalt sulphide method, it has been possible to locate with certainty any air which is in gaseous connexion with the exterior, to distinguish films of free air from air within a porous solid, and to gain some indication of the degree of porosity of such solids.

Much remains to be done in this field, but some provisional conclusions can be put forward. Special pneumatic arrangements in the egg are common but not universal. In *Locusta* and in the ladybird *Adalia*, in which the serosal cuticle provides the chief protective membrane of the egg, this is nowhere pierced by air-containing canals. A film of air collects beneath the dry chorion from which oxygen presumably diffuses through the general surface of the serosal cuticle.

In the eggs of *Syrphus*, laid exposed on the surface of leaves, the chorion is excavated like that of parasitic Diptera (Pantel, 1913) to form large cavities filled with air, and a film of air collects between the chorion and the chorio-vitelline membrane.

Apart from these examples the air in the egg is usually contained in porous membranes and is not in the form of free films. This porous substance may be distributed throughout the shell, as in the egg of *Psylla*, *Calliphora*, or *Drosophila*. The dorsal folds in *Calliphora* and the horns in *Drosophila* form local specializations of this general structure. In the Lepidoptera the chorion is lined with a thin layer of air-filled porous protein which communicates with the exterior through a limited number of channels likewise filled with porous air-filled substance.

Greater specialization is seen in the Hemiptera in which, as shown by Tuft (1950) in *Rhodnius*, the entry of oxygen is almost confined to the region of the cap. In *Oncopeltus* there can be little doubt that the 'sperm cups' of Leuckart (1855) are indeed the micropyles. But in the egg after laying, the central canal, which pierces the shell and presumably forms the micropylar duct, is completely blocked with cement; it is the spongy walls of the cups which convey oxygen to the thin porous layer that lines the shell. (In Pentatomids, according to Gross (1900), the spongy material completely fills the lumen of the 'sperm cups'.) In *Rhodnius* the true micropyles described by Beament (1947) are quite separate from the respiratory canals. These, the pseudomicropyles or Leuckart's canals, are filled with porous material and

conduct air to the porous 'resistant protein layer' which forms a thin pneumatic sheath inside the egg. The arrangement in *Cimex* is similar.

The most complex respiratory structures are those described in *Blattella* and *Dixippus*. The shell of *Dixippus* is full of cracks containing air. This air was thought by Leuckart (1855) to be concerned in respiration. But that is not the case; the true respiratory sheath is provided by the porous inner layer of the endochorion. This layer communicates with the exterior through a mass of spongy protein which fills the pore that is commonly regarded as the micropyle. Similarly in *Blattella*, the respiratory sheath is composed of a thin porous sheet containing air which lines the delicate chorion; and the chorion itself is made up of columns of spongy protein in a colourless matrix. This pneumatic system communicates with the exterior by way of a respiratory process at the upper pole of the egg and an elaborate stigmatic apparatus moulded in the crista of the oötheca.

As the eggs of *Rhodnius*, *Cimex*, *Pediculus*, &c., develop, their contents become somewhat reduced, and a spoon-shaped depression appears on either flank. The calcareous shells of Phasmids are too rigid to collapse in this way, but the inner sheet of the endochorion becomes progressively detached from the outer sheet and air collects between. Such air must play a part in respiration but it is not a necessary component of the respiratory apparatus.

The nature of the porous substance in these eggs and the mechanism by which it comes to contain air are problems requiring further study. In *Rhodnius* the 'resistant protein layer' which forms the porous membrane consists apparently of tanned protein containing no large amount of lipid material (Beament, 1946*a*). It is perhaps the tanning of this spongy substance which gives it the necessary rigidity. In *Dixippus* and in *Blattella* the porous layer seems to consist of lipoprotein.

The appearance of air in the system must be largely determined by the nature of the spongy material; for, as pointed out in an earlier paper on the appearance of air in the tracheal system (Sikes and Wigglesworth, 1930), if the walls of a cavity are waxy it is necessary to have only a minimal degree of supersaturation (such as might be induced by an imbibitional or secretory removal of fluid from the system (Wigglesworth, 1938)) to bring about the liberation of dissolved gases. If, therefore, an active absorption of water and the development of hydrophobe properties in the porous substance (the result perhaps of tanning or of wax secretion) were to occur simultaneously, the system would fill with gas.

The appearance of air is certainly not a simple matter of evaporation, for in *Drosophila* and in *Dixippus* it occurs (as Leydig (1867) observed in *Tachina*) while the egg is still bathed in fluid in the oviduct. It is of interest to note that 'pneumatization' of the eggs in parasitic Diptera often takes place at the same time as the darkening of the chorion (Pantel, 1913), a change which is known to reduce its hydrophil properties (Pryor, 1940). And in the experiment described above (p. 447) 'pneumatization' of the larger cavities in the chorion

in *Calliphora*, which does not occur naturally, can be induced experimentally by impregnating its substance with oil.

The existence of these air-containing layers is in no way incompatible with the water-conserving requirements of the egg-shell. The gas layer in *Rhodnius* lies outside the waterproofing wax, completely surrounding it. There is therefore no need to assume, as Tuft (1950) supposed, that the whole of the diffusing oxygen must cross the wax layer over the very small area at the ends of the pseudomicropyles. In fact, the presence of an enclosed layer of air outside a waterproofing wax increases considerably the impermeability of the system to water (Beament, 1945), while at the same time providing the largest possible area across which diffusion of oxygen may take place.

The efficiency of these mechanisms for meeting the respiratory requirements of the egg can be discussed quantitatively only in the case of *Rhodnius*, where some of the necessary data have been provided by Tuft (1950). Assuming that the *Rhodnius* egg is 3 mm. in length and 0·6 mm. in diameter, and consumes oxygen throughout its substance at a total rate of 0·3 c.mm. per hour, it is possible (using the formulae of Krogh (1920) for the rate of diffusion in tracheae, and of Hill (1928) for the rate of diffusion through living tissues) to calculate what would be the minimum diameter of a uniform trachea, running down the long axis of the egg, which would be able to supply oxygen in the quantity required.

In order to provide oxygen throughout the surrounding egg by diffusion alone, such a trachea, open at one end to the atmosphere, would require a diameter of the order of 8μ. Now the resistant endochorion which forms the spongy layer is of the order of 1μ in thickness. In cross-section it would therefore have an area equivalent to a trachea of about 400μ in diameter. If we assume that the air in this layer amounts only to one-hundredth of its volume, the remainder being the matrix of the sponge, this would reduce the equivalent trachea to a diameter of 40μ. Such a system would clearly be more than adequate to provide for the respiratory needs of the egg.

We are indebted to Mr. R. W. Horne of the Cavendish Laboratory for his helpful co-operation in taking the electron micrographs in Pl. I.

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DESCRIPTION OF PLATE I

Fig. 1. Electron micrograph of the untreated outer layer of the endochorion in the egg of *Dixippus*. Siemens electron microscope. 50 kV., × 8,000.

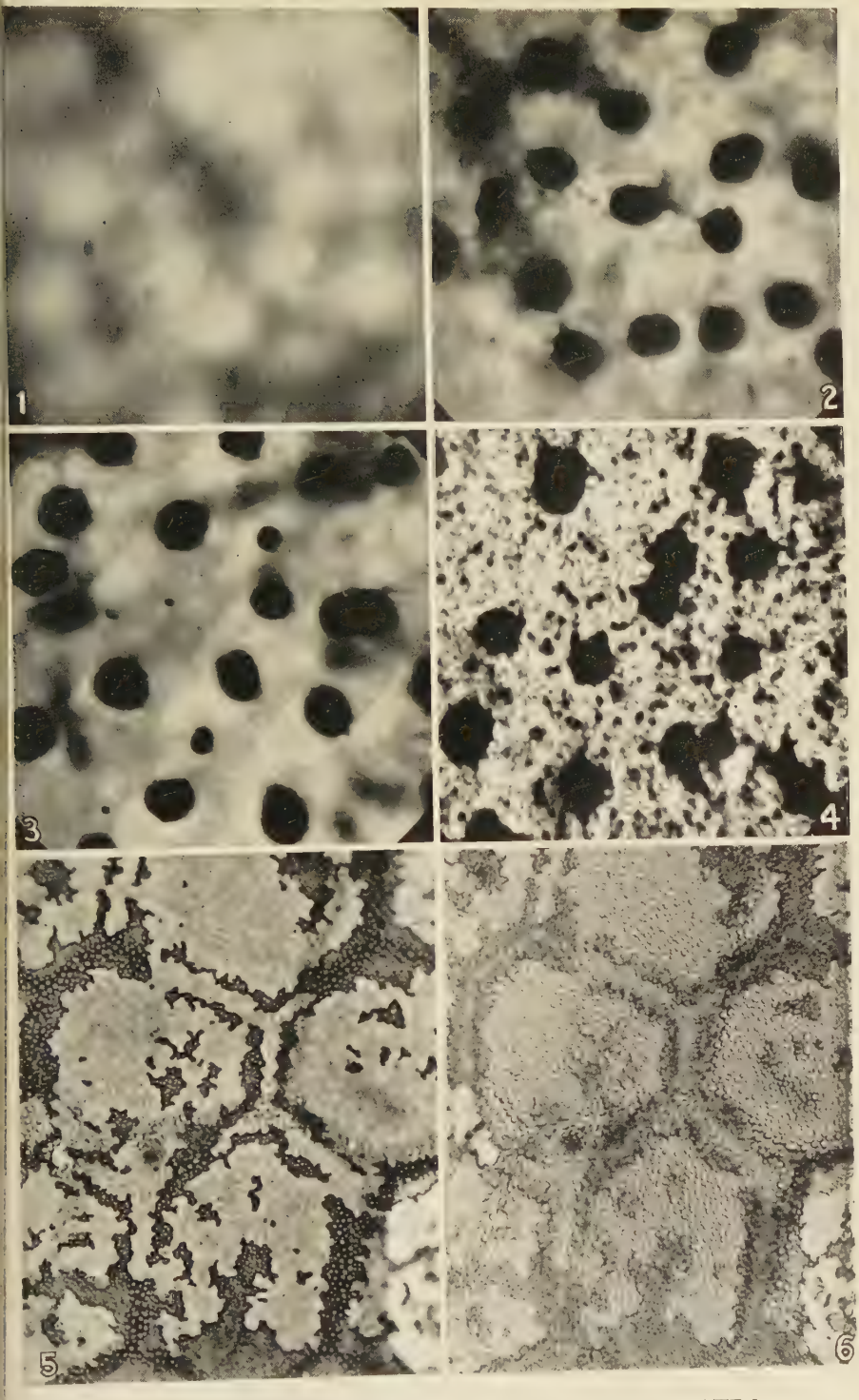
Fig. 2. Electron micrograph of the untreated inner layer of the same. 70 kV., × 8,000.

Fig. 3. Electron micrograph of preparation similar to fig. 2 after shadowing with gold and palladium at an angle of 45°. 70 kV., × 8,000.

Fig. 4. Electron micrograph of the inner layer of the endochorion in *Dixippus* after injection with lead naphthenate. 50 kV., × 8,000.

Fig. 5. Photomicrograph of two adherent choria from the oötheca of *Blattella*, mounted in glycerol. The air-containing layer that is in focus, with punctate depressions at the ends of the columns in the chorion, occurs chiefly along the boundaries of the follicular cell areas. × 700.

Fig. 6. The same preparation as fig. 5 focused at the level of the air-containing layer on the other chorion. This layer is continuous over several cell areas. It shows the larger size and wider spacing of the columns in the chorion along the cell boundaries. × 700.



V. B. WIGGLESWORTH AND J. W. L. BEAMENT—PLATE I

The Water Relations and Cuticle of *Paradesmus gracilis* (Diplopoda, Strongylosomidae)

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Summary

Water-loss in *Paradesmus gracilis* depends upon temperature and humidity, and is directly related to saturation deficiency. There is no evidence of any 'critical point' to indicate an epicuticular wax layer. Water is readily lost and taken up through the cuticle, the effect of the spiracles and of excretion being negligible. Despite great sensitivity to desiccation, there is nevertheless some degree of impermeability.

The cuticle is in many ways similar to that of an insect: it is composed of a 'cuticulin' epicuticle, a 'tanned' chitinous exocuticle, and a laminated endocuticle of two optically distinguishable layers. The outer endocuticle is strongly calcified. The cuticle is penetrated by pore canals and the ducts of dermal glands. The latter are concerned with the production of exo- and endocuticle, and the secretion of the polyphenols which tan the protein of the exocuticle. The double hardening is probably a specialized condition of millipedes.

Transpiration is almost quadrupled by extraction with hot, but not cold chloroform, as the exocuticle is impregnated with lipoids which reduce permeability. These are secreted by epidermal and dermal gland cells, and pass up the pore canals and gland ducts.

Contents

Introduction

INVESTIGATION of their sensory physiology and behaviour indicates that millipedes are extremely susceptible to the effects of desiccation, and that moisture is to them the most important environmental factor. The physiology of their water relations has therefore been considered in relation to the histology of their cuticle.

The integument of Arthropoda has been the subject of much recent research, and the extensive literature concerning the insect cuticle has been

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reviewed by Wigglesworth (1948*b*). The other classes have not been neglected, and recent work such as that of Yonge (1932), Drach (1939), and Dennell (1947*a*, *b*) emphasize the similarity of the integument of all arthropod classes. It consists normally of three layers, the most convenient terminology for which is that proposed by Campbell (1929) who refers to them as 'epicuticle', 'exocuticle', and 'endocuticle'.

The myriapod cuticle, however, has as yet been little investigated. The early accounts of Verhoeff (1902–25, 1926–8) do not include precise histochemical details, whilst that of Langner (1937) is also not comparable with recent work in other groups. Lafon (1943) distinguished two types of exoskeleton: 'organic' (Insecta, Arachnida, Chilopoda) and calcareous (Crustacea, Diplopoda). He did not believe that two types could occur in the same animal; but Dennell (1947*b*) has since demonstrated the presence of phenolic tanning in Decapod Crustacea, and established the homology of the crustacean cuticle with that of insects; while Hackman, Pryor, and Todd (1948) showed by means of the argentaffin reaction that phenolic substances occur in the cuticle of the Diplopod *Tachypodoiulus niger*. Edney (1949) demonstrated the absence of a wax layer in the cuticle of *Glomeris*, and Cloudsley-Thompson (1950) showed that an epicuticle of 'cuticulin' is present in both Diplopoda and Chilopoda.

Blower (1949), who has investigated the histology of the integument of Chilopoda and Diplopoda, has been kind enough to let me see his unpublished manuscript. He concludes that the cuticle in both groups is similar in that it consists of two optically distinguishable layers: an outer homogeneous, highly refractile, and usually pigmented exocuticle which appears to be a product of sclerotization as in insects, and an inner colourless, laminated endocuticle. Both layers are basically composed of chitin, and no evidence was obtained of an outer, non-chitinous epicuticle.

The principal occupants of the adult integument are the dermal gland cells which send ducts through the cuticle, opening on to the surface. These glands secrete the lipoid material which impregnates the exocuticle, where presumably it undergoes some change which reduces its staining properties with fat stains. It was pointed out that, despite the presence of lipoid materials in their cuticle, myriapods are very susceptible to desiccation.

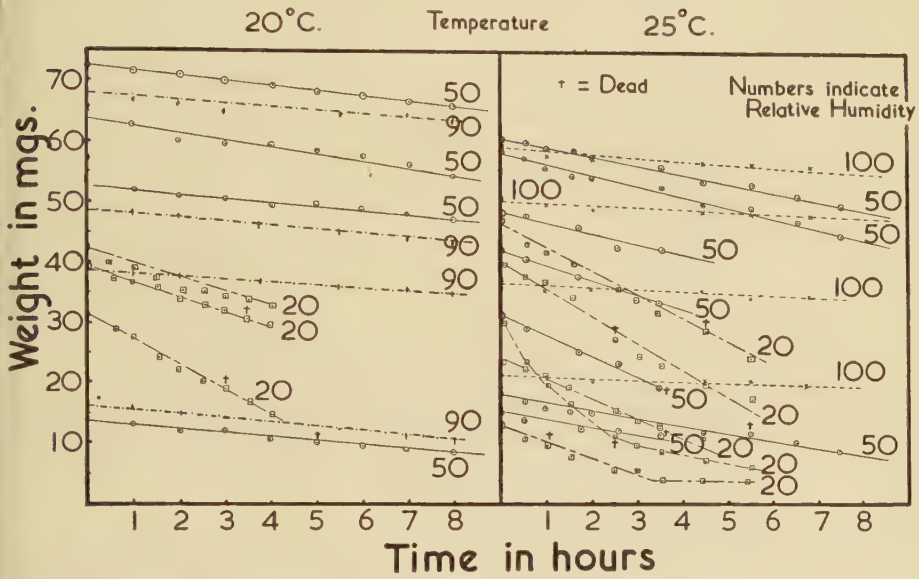
According to Blower, therefore, the cuticle of myriapods could, in some respects, be compared with that of the spider, *Tegenaria atrica*, in which Browning (1942) claims that there are but two layers of the integument: an exocuticle and an endocuticle which resemble the impregnated and non-impregnated cuticle of *Rhodnius* where variation in hardness is accompanied by the presence or absence of impregnation rather than by relative thickness. Browning found not only no epicuticle, but no pore canals, and no evidence of lipoids or waxes in the integument of spiders. He presumed that the exocuticle serves the function of the epicuticle at least in regard to permeability and desiccation. It has since been shown, however, that a 'cuticulin' epicuticle is found in most, if not all, arthropods (including spiders) whether additional

epicuticular layers are present as in insects (Wigglesworth, 1948*b*), and ticks (Lees, 1947), or not (Cloudsley-Thompson, 1950).

Temperature and Evaporation from the Cuticle

Preliminary Experiments

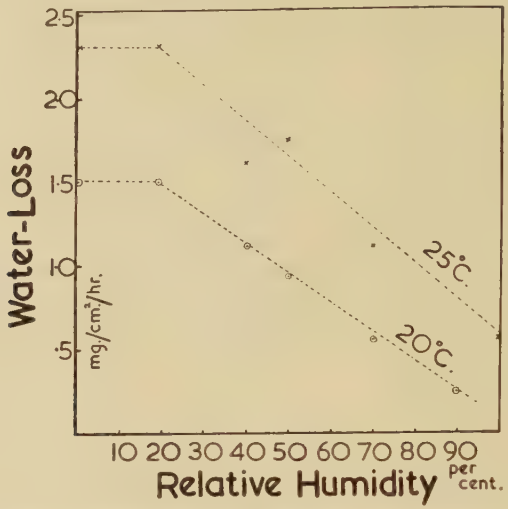
A number of living *Paradesmus gracilis* (Koch) were placed in an arena, the floor of which was composed of zinc gauze covered with fine voile. Sulphuric acid mixtures and distilled water were used to control the humidity at 50, 90, and 100 per cent. respectively (Buxton and Mellanby, 1934) at temperatures of 20° C. and 25° C. The millipedes were weighed at hourly intervals, and some of the results are given in Text-figs. 1 and 2. They show that water-loss is dependent not only upon the temperature and humidity at which the experiments were carried out, but also upon the initial weight and therefore surface area of the millipedes, from which it is inferred that evaporation takes place through the cuticle as well as through the spiracles. It is important to notice that the rate of water-loss remains almost constant from the outset of the experiment until it ceases altogether when the animals have lost about 50 per cent. of their weight and are completely desiccated. Possible differences in the initial state of the water-balance of various individuals do not therefore affect their rate of water-loss. Some slight decrease is to be expected under the influence of depletion of water in the body, as in *Agriotes* larvae (Wigglesworth, 1945), and in non-living membranes (King, 1944). This is usually very small, and is not apparent until the final stages of desiccation



Text-fig. 1. Relationship between water-loss and temperature and humidity.

are reached. The sensitivity of the animals to desiccation is indicated by the fact that they lose weight even over a bath of distilled water.

The fact that no significant loss in weight was noted in 10 *Paradesmus* which were kept in a medium of damp asbestos wool for 10 days, during which time a colourless liquid was excreted in place of the usual black faecal pellets, suggests that the effect of starvation is negligible.



Text-fig. 2. Relationship between rates of water-loss and relative humidity at different temperatures.

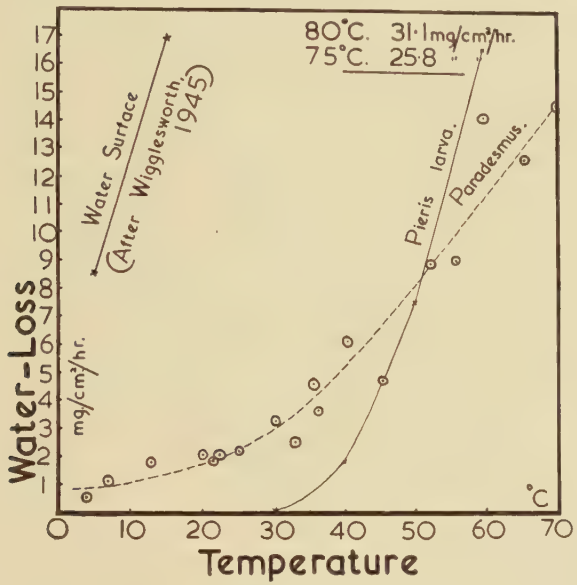
Effect of Temperature on Transpiration

The rate of water-loss per hour in dry air was ascertained by suspending weighed millipedes over anhydrous phosphorus pentoxide in conical flasks immersed in a water bath; the rate of loss being expressed as mg./cm.²/hr.

Estimation of effective surface is even more difficult in a millipede than in an insect; for not only are there innumerable fine irregularities, but the cuticle is calcified and cannot be spread out on squared paper. Instead, the relation between the width of the dorsal surface and that of the remaining lateral and ventral surfaces was measured on an enlarged photograph of a transverse section, by means of a map measurer (= 0·035). From this, the total surface area (S) was obtained by measuring the length and breadth of a millipede of known weight, and calculating the approximate surface area of the 62 legs. From this figure the value of k in the formula $S = k \times W^{\frac{2}{3}}$ was found, and the surface area of any other member of the same species could be calculated from its weight (W). The value of k employed = 15, which compares with *Agriotes* larva, 11·0; *Pieris* larva, 9·8; and *Tenebrio* larva, 8·4 (Wigglesworth, 1945). The value of k need be only approximate because specific differences in evaporation are so great even between different species

of insects, that an error of 50 per cent. in the value of k will not affect the conclusions drawn (Wigglesworth, 1945).

The results obtained are shown in Text-fig. 3, where water-loss per hour has been plotted against temperature. Each point represents a mean value of four individuals. At temperatures over 50° C. the water-loss was measured over a period of ½ hour only. Loss from an open water surface, and from *Pieris* larvae (from Wigglesworth, 1945) are added for comparison. They



TEXT-FIG. 3. Rate of evaporation of water at different temperatures.

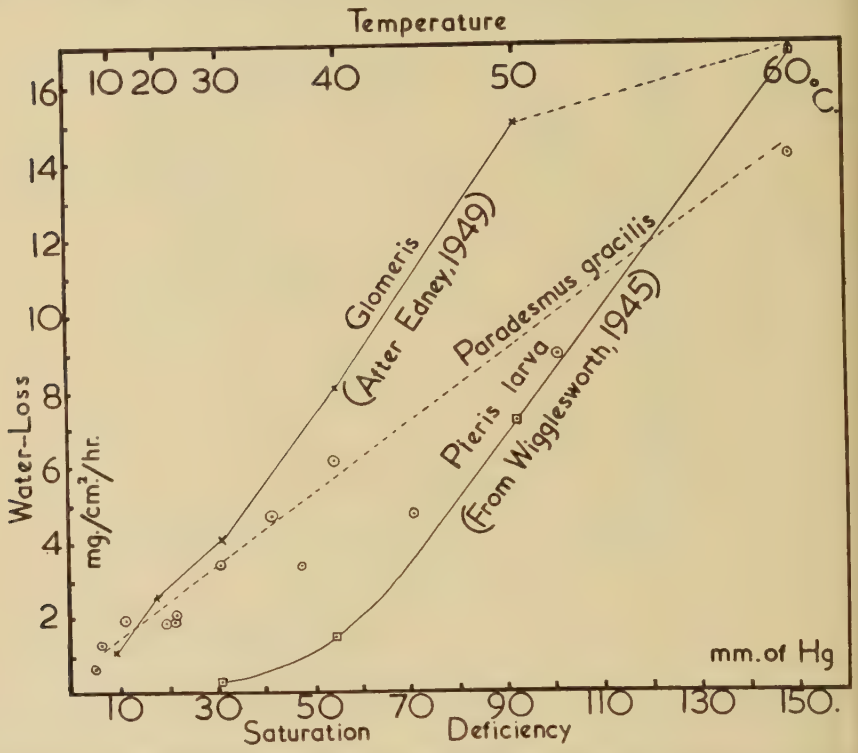
indicate that although *Paradesmus* is very susceptible to desiccation when compared with insects, the cuticle nevertheless possesses a certain degree of impermeability. No significant difference was noted between the water-loss of recently moulted, pale and more mature, darker individuals.

The absence of a critical point, and therefore of a wax layer, is seen even more clearly when water-loss is considered in relation to temperature and saturation deficiency (Text-fig. 4), where figures for *Glomeris* (from Edney, 1949) and *Pieris* larva (based on Wigglesworth's 1945 data) are added for purposes of comparison. The direct linear relationship is clear evidence of the absence of any discrete wax layer showing a critical temperature effect.

Water Excretion and Uptake

Effect of spiracles. In view of the impracticability of blocking the spiracles in the experiments outlined above, not only on account of their large numbers but because of the hard and shiny surface of the cuticle, it was thought possible that this difficulty might be overcome by carrying out experiments on living millipedes both in air and in an atmosphere containing a proportion

of carbon dioxide gas. Wigglesworth (1935) showed that in insects, concentra tions of CO_2 above 2 per cent. cause the spiracles to remain permanentl open; and it seemed possible that below the critical point of a wax layer, i present, and at all temperatures if not, animals in flasks containing CO_2 would lose water more rapidly than controls. No difference was noted, however after forty comparisons; and it was concluded that, if the spiracles do respon



TEXT-FIG. 4. Relationship between rate of water-loss and saturation deficiency at differen temperatures.

to carbon dioxide, the water-loss takes place so readily through the cuticle a all temperatures that the effect of the spiracles is negligible. Removal of th legs caused a marked increase in the rate of water-loss.

Effect of Excretion. Under normal conditions millipedes excrete moist blac faecal pads. When kept under conditions of starvation, a colourless liquid i excreted. At higher temperatures they may be observed drinking from drop lets of condensed water. When the mouth, anus, or both were blocked wit cellulose paint, the gain or loss in weight per sq. cm. of surface area on alter nate exposure to moist, and to dry surfaces in dry air, did not differ signifi cantly from that of control animals. It was concluded, therefore, that althoug millipedes may drink and normally excrete water, moisture is absorbed an lost so readily through the cuticle that the effect of the former is masked.

The Structure of the Cuticle

eneral

The sclerites of millipedes are dorsally telescoped into one another when e animals are extended, but are placed more or less end to end when in piral reflex. Consequently in transverse sections, the posterior half (metazo-te) of one segment is seen to lie outside the prozonite of the succeeding gment, and the arthrodial membrane can be seen connecting the two. Hairs ay be present, and are particularly numerous in Polyxenidae (Text-fig. 7). he cuticle of a diplopod as seen in transverse section consists of two layers: outer, homogeneous highly refractile exocuticle, and an inner colourless minate endocuticle. The endocuticle consists of two regions which can distinguished optically and chemically: an outer, translucent layer with osely spaced, ill-defined horizontal striae, which is impregnated with cal-um salts (Lafon, 1943); and an inner transparent and slightly refractile yer with fewer conspicuous, well-spaced horizontal striae (Blower, 1949). Langner (1937), too, describes two endocuticular layers: an outer 'Platten-hicht', and an inner 'Balkenschicht', and Verhoeff (1902–25) mentioned an ndocuticle of two layers. Blower (1949) suggests that the presence of an utermost 'epicuticle' (Grenzhäutchen) as recorded by Fuhrmann (1921) id Verhoeff (1902–25) in Chilopoda, and by Langner (1937) in Diplopoda, ased on staining reactions and appearance alone, can be attributed to optical fects and possibly a difference in the extent of sclerotization.

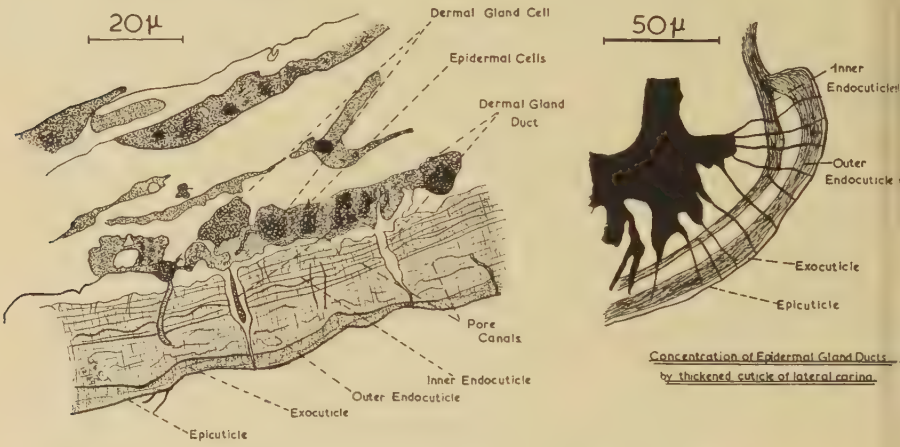
Histological methods. Millipedes are difficult to section on account of their xtremely hard, calcareous cuticle, and soft internal anatomy. Satisfactory sults were obtained, however, using ester wax. The animals were chloro-rmed for a few seconds, straightened with needle and brush, and placed in lenning's fixative. After 24 hours they were transferred to 70 per cent. coholic iodide, and left overnight. Next they were washed thrice in 90 per ent. alcohol, and placed in cellosolve. The following day they were washed wice in cellosolve, and left in an oven at 50° C. in a mixture of 50 per cent. ellosolve, 50 per cent. ester wax. Next morning they were transferred to ster wax which was replaced in the evening. After a week in this, they were mbedded, cut in 8 μ serial sections, and stained with Mallory's triple dye. n other cases normal wax sections were cut, but this involved considerable astage.

Dermal gland cells. In *Paradesmus*, gland cells are more numerous where he cuticle is thicker, for example, at the lateral carinae (Text-fig. 5). In entral areas the exocuticle is very thin or absent, and the gland cells and ucts are present in very small numbers, or else absent too. The cuticle of *Glomeris* is much thicker than that of *Paradesmus* (50 μ instead of 20 μ pprox.), and the dermal gland ducts can be seen in great numbers (Text-fig.). Owing to pigmentation, their path cannot easily be seen through the exo-uticle, but they appear to project beyond this, presumably on account of xcreted materials deposited at their openings.

Pore canals. Blower (1949) was unable to distinguish pore canals becaus these are usually not visible in histological sections. They can readily b seen, however, in air-dried wax sections, in frozen sections, and occasionall in sections stained with Heidenhain's iron haematoxylin, and are shown i Text-figs. 5, 6, and 7, leading from the epidermal cells to the outer surfac of the exocuticle.

Experimental

Effect of liquid paraffin. Liquid paraffin has a similar effect on the cuticl of millipedes as of insects (Hurst, 1940; Wigglesworth, 1941, 1942). Withi



TEXT-FIG. 5. Sections of cuticle of *Paradesmus gracilis.*

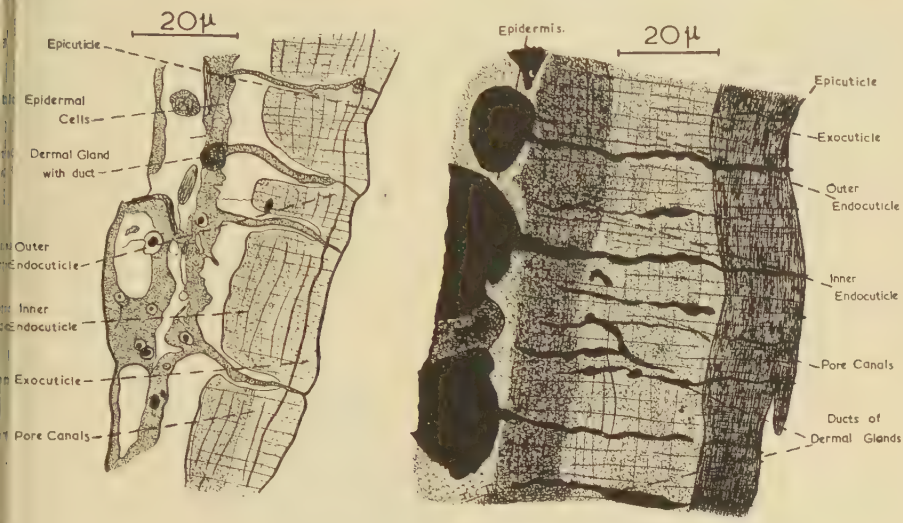
15 minutes of submersion, small droplets of water appeared all over th antennae of *Blaniulus*, and after 20 minutes all parts of the body were covered An hour and a half later, the animal was completely coated with fine bubbles The same process occurred, but more slowly and to a lesser degree, in *Para desmus*. The oil presumably dissolves in the lipoids which impregnate th cuticle, and so displaces the water in its deeper layers.

Inert dust abrasives. The rate of water-loss per sq. cm. at various tempera tures did not differ significantly from that of controls in 28 *Paradesmus* whic had been kept in an abrasive medium of damp alumina dust for several day (cf. *Rhodnius*; Wigglesworth, 1945). Hence there can be no epicuticular wa layer to be abraded.

Argentaffin reaction. Both in controls and in millipedes rubbed with alumin dust, the only parts stained black were the openings of the ducts of the derma glands. Blower (1949) obtained similar results, and explained them b suggesting that the outer layer of the exocuticle is inert 'sclerotin' which pro tects a reactive region of incipient 'pro-sclerotin'. However, polyphenols fre quently do not reduce silver oxide solution unless they are fresh, except i sections where a vastly increased reactive surface is exposed. Similar result

re obtained in the epicuticle of newly moulted insects before tanning has aken place (Wigglesworth, 1947), but in this case the polyphenol layer has ot yet been formed. In sections the exocuticle only can be seen to reduce o per cent. silver oxide: the epicuticle cannot be distinguished. Hackman, ryor, and Todd (1948) obtained similar results with *Tachypodoiulus* although hey designated the exocuticle as 'epicuticle'.

Effect of wax solvents. Although no epicuticular wax layer has been demonstrated in *Paradesmus*, it was thought possible that the lipoids which evidently



TEXT-FIG. 6. Sections of cuticle of *Paradesmus* and *Glomeris*.

impregnate the exocuticle might be removed with chloroform, which is a good general solvent for oils and waxes. Batches of four millipedes were exposed to chloroform vapour and extracted with chloroform at different temperatures before their rate of water-loss at 30° C. was measured. Half an hour was allowed to elapse after extraction to ensure that every trace of chloroform had evaporated, before weighing. The results are summarized in Table I. Comparative figures for *Rhodnius*, calculated from Wigglesworth's (1945) data, suggest that the wax is more easily removed from the cuticles of insects than of millipedes.

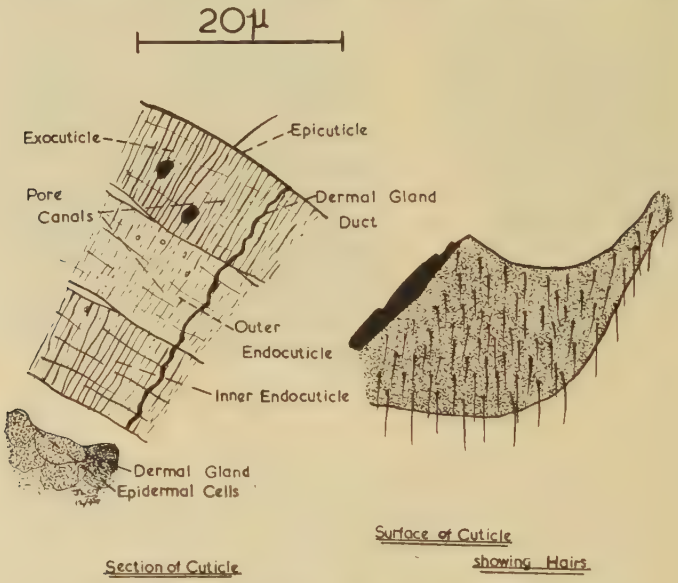
Chitosan test. This was positive to exocuticle and endocuticle of *Paradesmus gracilis*.

Lipoid colouring-agents. A number of *Paradesmus* were embedded in gelatine using Baker's method (Pantin, 1946); sectioned with a freezing microtome, and coloured with osmium tetroxide, or sudan black and Mayer's carmalum. There was some evidence that the pore canals and epidermal gland ducts took up the stain. Most of the exocuticle appeared to be stained also. No results were obtained with sudan IV.

TABLE I. *Loss of Water in mg./cm.²/hr. at 30° C. from* Paradesmus *and* Rhodnius

	Paradesmus	*Rhodnius*
A. Dead, normal controls	3·0	0·0001
B. After exposure to chloroform vapour for 1 hour at 20° C.	3·0	0·0009
C. After extraction with chloroform for 15 min. at 20° C.	3·0	0·13
D. After extraction with chloroform for 15 min. at 50° C.	10·7	1·7

Blower (1949) also found some evidence that in *Schizophyllum* the derma gland ducts, and the epidermal cells in the neighbourhood of the derma glands, were stained. He believed that lipoids passed up the gland ducts Langner (1937) claimed that the epidermal glands and their ducts staine



TEXT-FIG. 7. Cuticle of *Blanius* (section) and *Polyxenus* (surface, showing hairs).

with sudan III in *Sphaerotherium*. She did not record any staining of the exocuticle, but Blower suggests that perhaps this was because sudan III is less efficient than sudan B.

Sodium pyrogallate. The presence of calcium was ascertained by placing frozen sections in alkaline pyrogallol (Lison, 1936). The calcium replaces the sodium ions *in situ* as insoluble brown calcium pyrogallate. Calcification appeared to be restricted chiefly to the outer endocuticle, a result in agreement with that of Blower (1949). Incineration of sections at 600° C. and the action of dilute mineral acids lend support to this view.

Concentrated mineral acids. The calcified outer endocuticle dissolves rapidly in concentrated hydrochloric acid, followed by the inner endocuticle. The

ore canals are more resistant, and their fine filaments take longer to dissolve. he brown exocuticle does not dissolve, probably because it is tanned. It is luble, however, in chlorated nitric acid, leaving a thin colourless epicuticle hich dissolves on heating, forming oily droplets. It is insoluble in thioglu- nic acid. An epicuticle has not previously been conclusively demonstrated myriapods. Similarly results were obtained with *Blaniulus* and *Glomeris*. he thickness of the epicuticle does not exceed 1 μ: it is quite invisible in ost histological sections, which is why it has not previously been discovered ith any certainty (Cloudsley-Thompson, 1950). An epicuticle is easily seen, owever, in sections of the large Natal species *Doratogonus setosus* (Vog.).

Penetration of stains. A simple technique, based on Beament's (1946, 1948, 949) work, was devised to investigate the presence of wax in the cuticle. iving millipedes were beheaded, and their posterior segments and alimen- ary canals removed. The posterior ends were sealed with paraffin wax and he body-cavities filled from the head end, by means of a fine waxed pipette, ith Delafield's haematoxylin. These openings were then sealed with paraffin wax also, and the animals left in water for 3 days. Other animals were treated n a similar manner but filled with water and left to soak in stain. Both sets were then embedded in wax, and sectioned. In neither case was any part of he cuticle stained. The experiment was repeated after fixing with trichlor- cetic acid, and it was found that all layers of the cuticle took up the stain, particularly those of the endocuticle.

Discussion

Transpiration experiments show that water-loss depends upon temperature nd humidity, being directly related to saturation deficiency. There is no evi- dence of any 'critical point' which would indicate the presence of an epicuti- cular wax layer as obtains in insects and ticks; and water is readily taken up and lost through the cuticle, possibly via the pore canals, the effect of the spiracles and of excretion being negligible. Although the sensitivity of the animals to desiccation is so great that they lose weight even over a bath of distilled water, the cuticle nevertheless possesses a certain degree of impermeability.

Histological experiments show that the cuticle of *Paradesmus* is in many ways similar to that of an insect, and is penetrated by pore canals and the ducts of dermal glands. There is an outer 'cuticulin' epicuticle, an amber- coloured exocuticle, and an endocuticle composed of two optically dis- tinguishable layers, the outer of which is strongly calcified. Both exo- and endocuticle are chitinous: the former contains polyphenols and is 'tanned', as in insects. The contents of the gland ducts reduce silver oxide, suggesting that the dermal glands are concerned with the secretion of polyphenols. At the same time, concentrations of dermal gland cells when the cuticle is thickened indicate that these are also concerned with the secretion of exo- and endocuticle.

Hardening both by tanning of the exocuticle and by calcification of the outer endocuticle explains the extreme hardness of the diplopod integument.

It is possible that the condition is a primitive one since both the two methods of hardening employed by other arthropods are used; but since there is little phenolic tanning in Crustacea it would seem more likely that the millipedal condition is a specialized one.

The absence of an epicuticular wax layer is no doubt correlated with the humid environment in which the creatures live. At the same time the cuticle is impregnated with lipoids which reduce permeability to some extent. Transpiration is almost quadrupled by extraction with hot but not cold chloroform, hence these lipoids are more difficult to remove than those of the insect epicuticle. They appear to be secreted both by epidermal and dermal gland cells, and pass up the pore canals and gland ducts. It has not been possible to obtain suitable material for studying the moulting cycle, and thereby proving the above hypothesis.

Acknowledgements

My sincere thanks are due to Dr. V. B. Wigglesworth, F.R.S., under whose supervision this work was carried out, and to Drs. J. W. L. Beament, E. Bursell, and M. G. M. Pryor, who have offered helpful suggestions on various points.

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Aggregata leandri n.sp.

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SUMMARY

The schizogony of a newly discovered *Aggregata* from the prawn, *Leander squilla* L., is described, but its sporogony, expected in *Octopus vulgaris*, could not be found in the one specimen examined.

THE life-history of *Aggregata* was the subject of much research and controversy until 1914, when it was, I think, universally acknowledged to be a Coccidian (Dobell and Pixell Goodrich: references to early literature will be found in the latter). This Coccidian is exceptional in having a schizogonous stage in a Crustacean and sporogony in a Cephalopod. Even now the only species of which the complete life-history is known is *A. eberthi* with schizogony in a crab *Portunus* and sporogony in *Sepia officinalis*.

In April 1939 H. G. Callan working in Naples obtained from the northern end of Mergellina harbour some prawns, *Leander squilla* L., containing cysts, and kindly sent me some, beautifully fixed in a Bouin, sea-water, and iodine mixture.

On examining sections of the intestines and attached cysts they proved to be stages of an *Aggregata* undergoing schizogony. A few young trophozoites had already pushed their way through the epithelium and basal membrane and older ones had rounded off and were protruding into the haemocoel of the *Leander*. Some of these still had only the single trophozoite nucleus, but in others this had divided and the nuclei were arranging themselves round the periphery. In cysts still more advanced the cytoplasm had become folded to increase further the edges along which the multiplying nuclei could arrange themselves. The cysts were all suspended from the gut by one or more layers of peritoneum and full-grown ones measured up to 200 μ in diameter. In these the merozoites were often free and apparently all alike, about $10 \times 2\,\mu$, with the nucleus approximately $4 \times 2\,\mu$ close to the proximal end. In fact, all the stages resembled very closely those drawn by Geoffrey Smith (1905, Figs. 12 and 13*a*) for *A. inachi* from *Inachus*, the Spider crab, and also Léger and Duboscq (1903, Figs. 5 and 6) for *A. vagans* from *Eupagurus*, although at that time they were described as sporozoites of a schizogregarine.

It will be convenient to call this newly discovered *Aggregata* from *Leander* *A. leandri*, until we can complete its life-history by finding its other host. This seems likely to be the common *Octopus* which is, I was told at Naples, the only cephalopod which occurs in Mergellina harbour. In this connexion it may be worth while briefly recording a negative experiment I carried out

in Naples this March while looking for missing stages in life-cycles of some other protozoa.

Dr. Dohrn kindly arranged for the large tanks in my laboratory to be plentifully supplied with *Leander squilla* from Mergellina harbour before my arrival, and I kept an *Octopus vulgaris* from that harbour with some of them for 10 days and *Sepia officinalis* with others also only for a short time.

On examination before having to leave Naples, none of them proved to be infected with *Aggregata*—not even a single prawn, in which cysts, if present, can be seen through the transparent body-wall.

The remains of many softened exoskeletons of prawns were found in the intestine of the *Octopus* but no trace of an *Aggregata*. This was disappointing, for it was from this common *Octopus* that Schneider (1875) had first discovered *Aggregata* (*Benedenia*) *octopiana*. Also Dobell (1925) recorded that in Naples in 1908 he found 8 out of 8 infected. In addition to this the latter found 73 out of 73 of his *Sepia officinalis* contained *A. eberthi* and I had found from 1910 to 1914 that 100 per cent. were infected at Banyuls as well as in the Plymouth region and the Solent. On this occasion the only parasites found in my two *Sepia* were huge infections of *Dicyema* in the kidneys.

Up to the present, from the many Cephalopods investigated, only *Sepia officinalis* and *Octopus vulgaris* are known to be hosts of *Aggregata*, the latter probably containing more than one species, as held by Moroff and admitted by Dobell (1925, p. 20).

On the other hand, many Crustacea have been found to harbour the schizogonous stages, as follows:

1. *A. eberthi* Labbé from *Portunus depurator* L. and some other species of this crab.
2. *A. coelomica* Léger from *Pinnotheres pisum* Penn.
3. *A. conformis* Dies. from *Pachygrapeus marmoratus* F.
4. *A. vagans* L. & D. from *Eupagurus* (3 species).
5. *A. inachi* G. Smith from *Inachus* (2 species).
6. *A. leandri* n.sp. from *Leander squilla* L.

It is possible that all the so-called species 2–6 above may undergo their sporogony in *Octopus vulgaris*.

Dobell (1925, p. 30) gave some interesting notes on his and others' difficulties in infecting many Crustacea with spores from *Sepia* and came to the conclusion that a particularly thick basal membrane may prevent freed sporozoites passing into the haemocoel to complete their schizogony. It seems well in this connexion to point out that active sporozoites in the intestine are not necessarily a sign of infectibility of the host, for the spores would probably have opened and liberated active sporozoites when mounted *in vitro* in any suitable intestinal fluid, as do those of fowls and rabbits (Pixell Goodrich, 1944).

In *Eupagurus* and *Inachus* polycystid gregarines were also recorded from the intestine and this perhaps in part accounted for the learned French proto-

ologists, Professors Léger and Duboscq, connecting the life-history of *ggregata* with schizogregarines.

The polycystid gregarines after their early association probably remain in e alimentary canal of their host until they begin to encyst and then pass out complete their sporogony in the sea, or possibly in another marine animal, *Porospora* is said to do in a Mollusc.

Acknowledgements

I should much like to take this opportunity of expressing my thanks to r. Dohrn and the staff at the Stazione Zoologica for their courtesy and help uring my visit to Naples. I am also very grateful to the Council of the British ssociation for allowing me to use their Table at the Stazione. My thanks are so due to Professor A. C. Hardy, F.R.S., in whose Department here in xford much of the work has been done, and to Professor H. G. Callan for ne trouble he took in fixing and sending the material.

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Sporozoa of *Sipunculus*

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SUMMARY

The gregarines of *Sipunculus*, so often recorded during the last hundred years, prove to belong to two or more species of the genera *Urospora* and *Lithocystis*, and further information especially about the gametes and zygotes is much needed.

The structure of the spores, the most diagnostic feature of gregarines, forces one to recognize three species, viz. *Urospora légeri*, *U. hardyi*, and *Lithocystis lankesteri*, as distinct from the original *U. sipunculi* Köll.

MORE than a hundred years ago Kölliker (1848) described a parasite of the coelom of *Sipunculus nudus* from Naples which he called *Gregarina sipunculi*. He appears only to have examined a few specimens of *S. nudus*; one contained trophozoites described as generally roundish or pear-shaped, smooth, and active, later becoming associated and approximately spherical (see his Figs. 1–3); another specimen had many cysts near its posterior end, each containing numerous oval spores with thread-like tails rather more than half as long again as the spores themselves (his Fig. 4*a*).

There is little doubt that this parasite should be put in Schneider's (1875) genus *Urospora* as *U. sipunculi* Köll., in spite of its being less elongated than most *Urospora*, and, as I pointed out (1915, p. 99), it requires much further study. Lankester working in Naples in the winter of 1871–2 on the anatomy of *S. nudus* had found numerous specimens infected with gregarines, and some of the spores drawn by him (Fig. 6 *b*, *c*), probably represent the above species. However, he described, p. 348, another spore found on various occasions, with a long 'shred-like filament dependent from one extremity, which is perfectly motionless'. This gregarine, which I was able to examine alive in Naples, 1929 (Text-fig. 3), should belong almost certainly to the genus *Lithocystis* (Giard 1876) and I propose to call it *L. lankesteri* for the present. Lankester said that the spores were preceded by spherical corpuscles; these were undoubtedly gametes or zygotes and presumably motionless, but apparently neither he nor any subsequent worker has been able to examine them alive, so that we do not know for certain that they are motionless, as are those of other *Lithocystis* (emended characteristics, Pixell Goodrich, 1915, 1925).

From January to June 1921 A. C. Hardy was working in Naples, and some *S. nudus* he examined were infected with parasites. Among these were motile trophozoites of two distinct gregarines—one with a spiny cuticle (Text-fig. 1, A and B), the other smooth and squirming (Text-fig. 2); but, as no spores were

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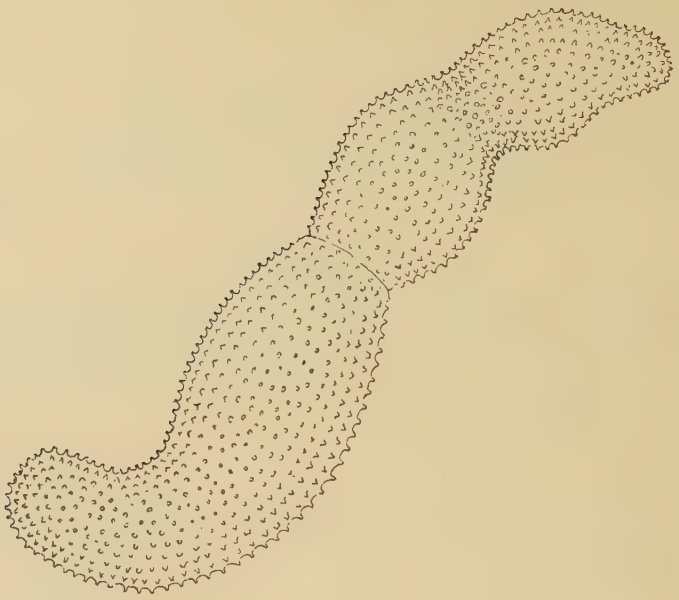

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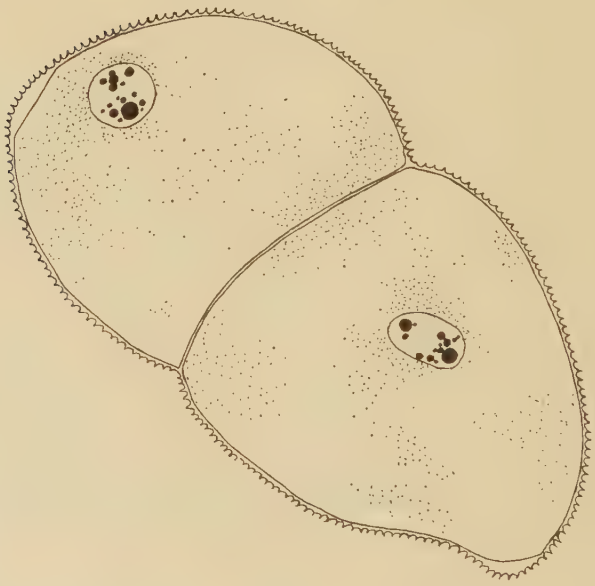
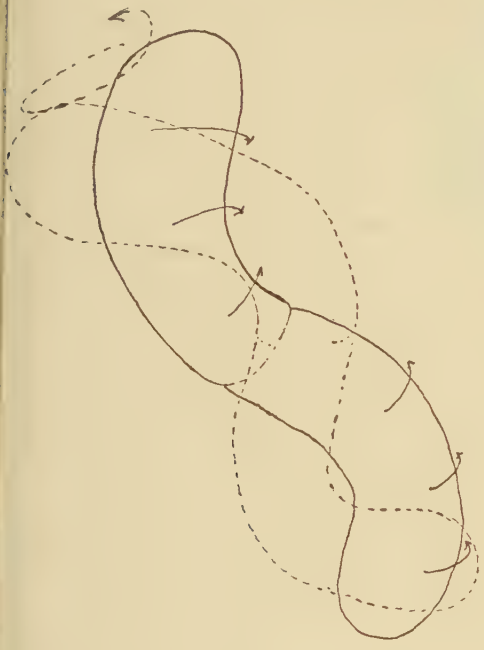


B

Text-fig. 1. Associated pairs of *Lithocystis lankesteri* n. sp., showing processes on cuticle. ×40 approx. A, alive, after removal of many coelomic corpuscles; B, fixed in Bouin's mixture and stained with iron haematoxylin. A.C.H. del.

und, the two species could not then be identified. However, from May to eptember 1924, he had some two dozen specimens of *S. nudus* fixed in rmalin sent separately from Naples to him in England. These he has ecently kindly handed over to me together with the good preparations and rawings he made of trophozoite stages while in Naples.

About one-third of the preserved specimens have proved to be slightly in-ected and I have been able to find spores of *L. lankesteri* (Text-fig. 3) and of one



TEXT-FIG. 2. *Urospora hardyi* n.sp. ×40 approx. Diagram to illustrate movements. 'S' shape retained throughout but the 'S' rotated slowly, about 10 revolutions to the minute; in addition to this, it rotated more quickly, 30 times a minute, about its own curved longitudinal axis. A.C.H. del.

other gregarine, a *Urospora*, which I propose to call *U. hardyi*, characterized by a spore with a very short tail (Text-fig. 4). These two gregarines I found also in living specimens of *S. nudus* in 1929. While in Naples in March and April of that year, my husband, E. S. Goodrich, noticed an infection in a *Sipunculus* he was working on, and passed it on to me. In all, 22 specimens, of which 8 were more or less infected with gregarines, were examined at that time, but all the hosts were very actively destroying their parasites, and nearly all cysts and some trophozoites had been engulfed in the brownish masses of phagocytes so often found in this and other invertebrates (Pixell Goodrich, 1916, p. 209). These masses could often be found in the angle between the retractor muscles and body-wall. In other specimens no traces of the whitish parasites could be found, and the masses were homogeneously brown and sometimes very large (5×2 mm. or more) and crowded together at the posterior end of the *Sipunculus*.

A small oval brown mass has been seen attached to the anterior region of the gut covered with a thick white homogeneous layer as well as a coating of

peritoneum. Sometimes the outer peritoneal covering of the gut had also a fringe of phagocytes (see encapsulated gregarines, p. 474 below).

This 1929 material was not therefore satisfactory for the study of parasites; in recently engulfed cysts the structure of the spore could be made out, but

TEXT-FIG. 3. Spore of *L. lankesteri* n.sp., × 3,000 approx., containing 8 sporozoites and large residuum.

in others even the spores were so necrotic as to be hardly recognizable. So far then the only species in addition to Kölliker's *Urospora sipunculi* that can be distinguished with any degree of certainty are the two following—the spores being, as usual in Sporozoa, of the greatest importance in establishing species.

Lithocystis lankesteri n.sp.

Endospore 12–14 μ long by 6–8 μ wide with thick refringent wall having a small plug of refringent material like the wall at the base of the funnel into which the ectospore is produced at one end. In both Lankester's and Kölliker's figures the whole funnel looks rather like a knob: it must be remembered, however, that ectosporal processes, both funnels and tails, are difficult to see unless fresh and stained (Stephens' ink being very convenient (Pixell Goodrich, 1915, p. 83)). When fixed and mounted unstained they are nearly

invisible. If folded and stained the funnel may appear like a group of spines as in Léger's description, p. 476 below. The long ribbon-like tail is 50–60 μ long (Text-fig. 3).

The spore contains, as usual in gregarines, 8 sporozoites and a residuum of highly refringent granules towards its centre: this mass breaks up when the spore becomes necrotic.

Owing to their relative abundance it seems likely that these spores come from the spiny paired trophozoites (Text-fig. 1), which so long as they retain their movements keep fairly free from attack by phagocytes. Only occasionally have a few phagocytes been seen pushing in between the spines. On the whole they seem to function like the much longer hair-like processes of the gregarines living in the blood-vessels of *Chiridota* (Pixell Goodrich, 1925). I cannot be sure whether this trophozoite common in our *Sipunculus* is the same as Lankester's (1872, Fig. 3) 'tuberculated' rectangular form with undulating movement like a planarian. Mingazzini (1891) described a trophozoite something like this under a new generic name and without figures, so far as I can make out, although Labbé (1899) mentioned some. Unfortunately, all Mingazzini says of the spore is that it had a 'sort of tail' and spherical nucleus. For the latter he no doubt mistook the residuum; this has often been done even recently by some workers. There have been indications in some of the necrotic cysts I have examined that there may be at least one other species: a spore with a short wide tail.

TEXT-FIG. 4. Spore of *U. hardyi* n.sp. with 8 sporozoites and residuum breaking up.

Urospora hardyi n.sp.

The other trophozoite that I have seen alive, and whose movements were so well described by A. C. Hardy (Text-fig. 2), would seem to have produced the spores with very short tails (Text-fig. 4), which are among his 1924 specimens and which I also found in Naples in 1929; they are, too, probably the same as those drawn by Lankester (1872, Fig. 6, *d*, *e*). The tails were 4–6 μ long, the whole spore only about 16 μ long. Ectosporal processes are not brittle, so there is no chance of the short tails having been broken. The ordinary thin-walled cysts vary in size from 0·5 to 2 mm. in diameter. When attacked by phagocytes, they undoubtedly shrink, but I have no definite evidence as to the specificity of size.

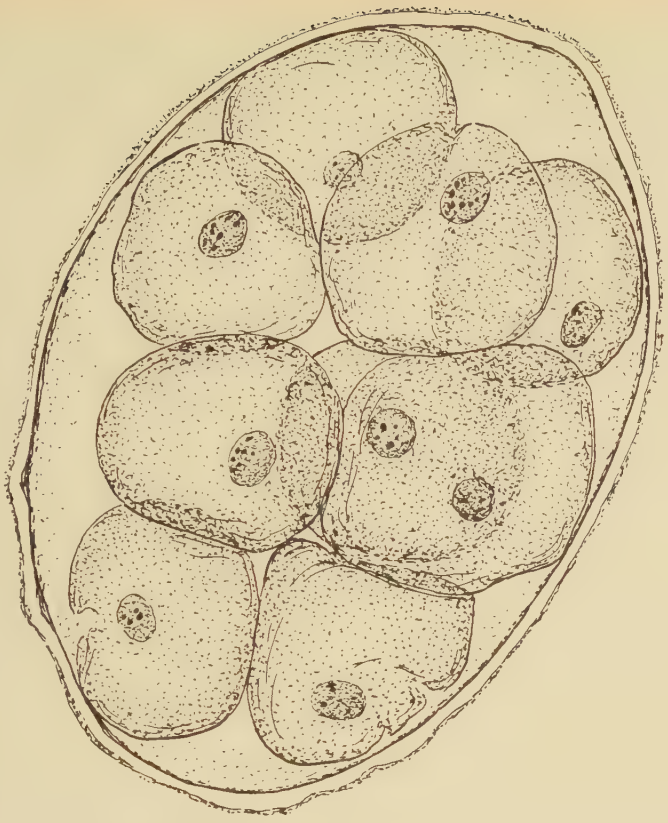
Any of the spores swallowed by the host no doubt open in the gut. I have seen there free forms not much longer than sporozoites (10 μ) and also young gregarines up to 40 μ long very like Lankester's (Figs. 7, 8, and 18), but could not say to which species of gregarine they belonged, nor at what stage each forced its way through the gut-wall into the coelom. Sometimes quite large spherical cysts containing paired trophozoites remain suspended by peritoneum from the gut and project into the coelom, especially in the neighbourhood of the rectal arborescent tufts. No doubt many of these do develop their

spores as Léger found with his specimens (p. 476 below), though this early encystment must do away with the motile stage in the coelom which is so usual in the genera *Urospora* and *Lithocystis*. On one occasion I found an active pair resembling *U. hardyi* rolling themselves up in a capsule consisting of a portion of such a rectal tuft which had become loose in the coelom. Unfortunately it was not possible to get these or other associated trophozoites to continue their development *in vitro*, although they may remain active in coelomic fluid or suitable saline for many hours. There is little doubt that this is due to the difficulty in keeping them aseptic, for in other gregarines with which I have experimented it has been equally impossible to get a pair to secrete their cyst when removed from the intestine or coelom, whereas after encystment further development can easily be observed *in vitro*, whether the cysts are removed with a pipette or naturally extruded (Pixell Goodrich, 1938, p. 119; 1949). In some *S. nudus* we have found a very unusual stage in the life-history of *U. sipunculi* where unassociated trophozoites either singly or in groups up to 9 become encapsulated, apparently while passing through the intestinal wall of the host (Text-fig. 5A). The wall of a large capsule may be as much as 100 μ thick and is often homogeneous, as are those enclosing single parasites where this smooth capsule wall is in contact with the cuticle of the gregarine: they may be covered and suspended for a time by peritoneum. Some of the larger capsules, after fixation and staining with haematoxylin, showed a layer or two of nuclei presumably of phagocytes inside a reduced homogeneous layer and occasionally phagocytes on the outside also. Further, the capsule wall sometimes appeared to be laminated or even fibrillated instead of smooth and homogeneous. Large capsules, from 1 to 5 mm. in diameter, were conspicuous objects free in the coelom. If slit open, the trophozoites, often polygonal in shape due to compression inside the capsule, would regain their rounded or pear-shaped forms, but showed no attempt at association. Each, as usual, had its large spherical nucleus with characteristically numerous caryosomes of various sizes. Unfortunately we have no reliable way of determining sex in these vegetative stages of gregarines. If they *should* be all of one sex in a capsule, that would seem to be a reason for the host trying to get rid of such parasites, which, having failed to find mates, were presumably destined to decay in its tissues.

Anyhow, it seems likely that the capsule is an autocyst secreted by the host to separate the parasites from its tissues, possibly after phagocytes had failed to do so in the ordinary way.

So far as our observations go, nothing of this sort has ever been described before in connexion with gregarines, though Kölliker (1848, p. 3) mentioned a 'Kapsel' containing many gregarines in a *S. nudus* from Naples examined by him in April, and Labbé (1899, p. 44) mentioned 'Kystes formés par l'association de 2–12 individus'.

In 1892 Léger gave an excellent description of another *Urospora* from a *Sipunculus* at Morgat in the Bay of Douarnenez (NW. France). He did not identify the *Sipunculus* but referred to them as 'ces énormes Géphyriens'.



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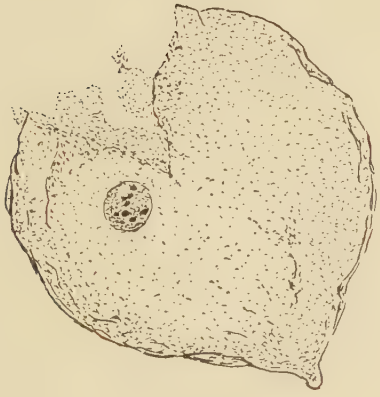

B

TEXT-FIG. 5. *Urospora sipunculi* Köll. Spherical nuclei with characteristically numerous caryosomes. A, capsule containing 9 trophozoites. × 28 approx. B, single crushed, pear-shaped trophozoite escaped from a capsule. × 40 approx. A.C.H. del.

The gregarines were suspended from the gut, sometimes in groups of two or three and covered with peritoneum—in fact the spores reached maturity in cysts up to 2 mm. diameter, which were still sometimes attached. Thus this *Urospora* appears to differ from most described species in having no motile trophozoite in the coelom. The spore also differed from those of Kölliker's *U. sipunculi*, to which Léger allied it, in having long filiform tails, 6 or 8 times the length of the endospore. The ectospore also was unusual in having longitudinal folds giving it a hexagonal outline in cross-section and a funnel appearing to have 6 spines owing to the prolongation of these folds (Léger, Pl. XX, Figs. 12–14). It would be well to rename it *U. légeri* to prevent confusion.

After writing the above and being very conscious of the incompleteness of our observations, it seemed advisable to make another attempt to find some of the required stages, so I flew out to Italy in March. Apparently *S. nudus* in good condition is now more difficult to obtain in the Naples region, and I could only examine 25 altogether. In only 2 were any gregarines to be found, and these were trophozoite stages not particularly wanted. This being so, it seems as well to publish this account of our observations in the hope that someone else will later find the missing stages. Marine invertebrates seem as liable to epidemics as are vertebrates. Both Enriques (1902) and Metalnikoff (1900) wrote long papers on *S. nudus* from Naples, without mentioning a gregarine, though the latter recorded an unknown ciliate.

Acknowledgements

I am very grateful to Dr. Dohrn and his Staff at the Stazione Zoologica for the trouble they took in obtaining these and other specimens for my work and for their courtesy and help throughout. I should also like to take this opportunity of recording my thanks to the Committee of the British Association for their kindness in allowing me to use their table at the Stazione during the weeks I spent in Naples. To Professor A. C. Hardy, in whose Department some of the work has been done, I am most grateful, and have made much use of his specimens, notes, and figures, five of the last being reproduced above.

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Alcian Blue 8GS: A New Stain for Mucin

By H. F. STEEDMAN

(*From the Department of Zoology, The University, Glasgow*)

SUMMARY

A new method of staining mucin is described. The stain used is alcian blue 8GS The method is rapid and easy in application. The results are clear and permanent.

THE disadvantages of most staining methods employed for the demonstration of mucin in histological sections of animal tissues are as follows:

1. Doubtful specificity. Not only mucins accept the stain; other elements such as nuclei, connective tissues, &c., may do so as well.
2. Loss of stain. In some cases where mucin is stained in a specific manner by dilution of the stain used, by adjustment of the pH of the stain solution, or by metachromatic properties of the stain, it is frequently difficult or impossible to dehydrate, clear, and mount the section without either immediate or later loss of the stain.
3. The stained mucin may have other stains added to it during additional operations.
4. The staining methods may be involved or may require the making-up of reagents which are often unstable.

'Alcian' blue 8GS has only one of these defects—it will not distinguish chondroitin sulphuric acid complexes from mucoitin sulphuric acid complexes. It therefore stains cartilage and mucin equally. Its advantages are that it is easy to apply; that it stains mucin clearly and conspicuously; that it combines with mucin in such manner that additional stains produce little or no alteration in colour. It reacts with mucin with such vigour that all common histological reagents fail to dislodge it, and only prolonged treatment with acid alcohol (2–24 hours) will reduce an overstained section to the required intensity. Once applied it will resist indefinitely water, alcohols, alkalis and hydrocarbons, and weak acid solutions for short periods of time. Preparations stained in 1947 and mounted in balsam, 'sira', and 'distrene' show no sign of fading.

Alcian blue 8GS is produced by Imperial Chemical Industries Ltd., to whom thanks are due for supplying dyes from time to time. It is a water-soluble precursor of the pigment monastral fast blue, into which it may be turned by action with alkalis. Industrially it is used in the dyeing of cotton.

It is not suggested here that alcian blue is a specific stain for mucin. Indeed, if staining is prolonged it will stain every tissue in a section except the nucleus.

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It is claimed, however, that if mucin is present alcian blue 8GS can be so used that only the mucin will be stained.

Tissues fixed in picric acid or mercuric chloride fixatives (Bouin, Susa, Zenker, &c.) give the best results. Formaldehyde material is not good.

Method

1. Bring paraffin wax or ester wax (Steedman, 1947) sections down to water in the usual way.
2. Stain with filtered 1 per cent. alcian blue in distilled water for 10–40 seconds. If longer times are given other tissues will take up the stain.
3. Rinse in distilled water to remove the excess stain solution.
4. Stain with haemalum 5–10 minutes.
5. Continue with the normal haemalum technique, counterstaining with eosin or whatever other additional stains may be required.
6. Dehydrate, clear in xylene, mount in balsam or any other resinous medium.

This method will stain any mucin a clear blue-green colour and the addition of the other two stains will in no way affect it. Where it is feared that an aqueous staining solution may dissolve imperfectly-fixed mucin, the ribbon staining method with ester wax sections may be employed.

Generally it takes so long using acid alcohol to remove excess alcian blue from a section that it is better to use the stain progressively as indicated, and the maximum time of 40 seconds is rarely exceeded with advantage.

Dilution of the stain to 0·1 per cent. or even to 0·01 per cent. does not appear to confer any particular advantage on the staining procedure.

The method has so far been used on a variety of vertebrate and invertebrate material without failure.

Should the use of stains in acid solution be contemplated after using alcian blue, two courses may be followed to counteract the slight destaining action of such fluids:

1. The section may be slightly overstained with alcian blue. This would mean 40–60 seconds in a 1 per cent. aqueous solution.
2. The section could be stained for the usual 10–40 seconds, rinsed in water, and taken up to alkaline 70 per cent. alcohol (pH 8 or over). Left in this solution for 2 hours or longer the bonds which make alcian blue a soluble dye are broken down and it becomes the insoluble pigment monastral fast blue, which will resist any further histological reagents. The longer the stained section remains in alkaline alcohol the more insoluble the stain becomes.

These modifications are not required when using dye solutions which are weakly acid, neutral, or alkaline.

It should be noted that while such dyes as thionin, methylene blue, toluidin blue, &c., give polychromatic effects in sections which contain mucin, macrophages, and tissue basiphils, alcian blue does not. It stains only mucin, and macrophages and tissue basiphils remain unstained. (Jorpes, Holmgren, and Wilander (1937) believe that the staining constituent of tissue basiphils is heparin.)

In aqueous solution alcian blue develops mould very rapidly. Thymol may be used to reduce the rate of growth, but even then filtration is advisable every 10 days. A freshly made solution stains best.

Alcian blue 8GS is a phthalocyanin dye and its chemical constitution is discussed by Haddock (1948).

ACKNOWLEDGEMENTS

Professor C. M. Yonge, F.R.S., has given continuous encouragement to this work throughout. Thanks for unfailing practical assistance are extended to Mr. A. McKinnon.

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